Thesis for doctoral degree (Ph.D.) 2010

Karolinska Institutet

200 1810 – 2010 Years

Thesis for doctoral degree (Ph.D.) 2010

THE INFLAMMATORY RESPONSE IN EXTRAVASATED LEUKOCYTES IN PATIENTS WITH CORONARY ARTERY DISEASE

Josefin Paulsson

Thesis for doctoral degree (Ph.D.)
2010

THE INFLAMMATORY RESPONSE IN EXTRAVASATED LEUKOCYTES IN PATIENTS WITH CORONARY ARTERY DISEASE

Josefin Paulsson

Karolinska Institutet

200 1810 – 2010 Years

THE DEPARTMENT OF MEDICINE
Clinical Immunology and Allergy Unit
Karolinska Institutet, Stockholm, Sweden

THE INFLAMMATORY RESPONSE IN EXTRAVASATED LEUKOCYTES IN PATIENTS WITH CORONARY ARTERY DISEASE

Josefin Paulsson

Karolinska Institutet

Stockholm 2010

ISBN 978-91-7409-812-9

Printed by
REPROPRINT AB
Stockholm 2010
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

EMBRACE LIFE

ABSTRACT

Coronary artery disease (CAD) is a manifestation of a chronic inflammation in the coronary arteries. The inflammatory process results in accumulation of monocyte derived cells and formation of atherosclerotic plaques in the intima of the vessel wall. Neutrophils are mainly associated with ruptured plaques and the precise role in CAD is not fully known. Extravasation into local inflammatory sites is coordinated by adhesion molecules and chemokines and transforms the leukocytes into activated tissue dwelling cells. The aim of this thesis was to investigate extravasated monocytes and neutrophils in patients with stable CAD. A skin chamber method was applied in order to induce a local inflammation from which extravasated cells were collected.

Paper I and II describes extravasated monocytes. Patients with CAD had a similar number of extravasated monocytes in the chamber exudate compared to healthy subjects. The expression of CD11b following extravasation was lower in patients with CAD compared to healthy controls. This might result in an increased retention of monocytes at a local inflammatory site. Other markers associated with monocyte extravasation, VLA-4 and CX_3CR1, were not altered. Extravasated monocytes were further analyzed for functional alterations. Markers associated with antigen presentation, HLA-DR and CD86, and binding of modified cholesterol, CD36 and scavenger receptor A1 (SR-A1) had an increased expression following extravasation compared to in circulation. Furthermore, the binding of acetylated low density lipoprotein (acLDL) increased following extravasation. Monocytes from patients and controls had a similar functional response. However, the chamber fluid from patients with CAD enhanced the expression of CD36 following *in vitro* stimulation of mononuclear cells.

Paper III and IV describes extravasated neutrophils. Extravasated neutrophils from patients with CAD had a significantly lower expression of CD11b and a lower production of reactive oxygen species (ROS) following stimulation compared to healthy controls. This might indicate a refractory stage following extravasation in patients with CAD. The gene expression in extravasated neutrophils was assessed by a gene array. A general induction in the IL-1 axis was seen following extravasation and was associated with an increased expression of chemokines. Expression of IL-1R on human neutrophils was confirmed with flow cytometry and electron microscopy and stimulation with IL-1 resulted in CCL and CXCL chemokine gene and protein expressions. Compared to healthy controls, extravasated neutrophils from patients with CAD had significantly increased expressions of CCL20 and CXCL2. This finding indicates that neutrophils may have an immuno-modulatory role at local inflammatory sites and that patients with CAD have a chemokine profile that could enhance the pathological processes in atherosclerosis.

The major findings indicate a potential mechanism for monocyte entrapment at local inflammatory sites. In addition, the local inflammatory milieu in patients with CAD might be pro-atherosclerotic. Neutrophils from patients with CAD had an altered responsiveness and could be refractory. Furthermore, neutrophils may alter the local inflammatory milieu by the production of chemokines.

PUBLICATIONS

I. Paulsson JM, Dadfar E, Held C, Jacobson SH, Lundahl J.
In vivo transmigrated monocytes from patients with stable coronary artery disease have a reduced expression of CD11b.
Clin Exp Immunol. 2008;153:196-204.

II. Paulsson JM, Held C, Jacobson SH, Lundahl J.
In vivo extravasated human monocytes have an altered expression of CD16, HLA-DR, CD86, CD36 and CX(3)CR1.
Scand J Immunol. 2009;70:368-76.

III. Paulsson J, Dadfar E, Held C, Jacobson SH, Lundahl J.
Activation of peripheral and *in vivo* transmigrated neutrophils in patients with stable coronary artery disease.
Atherosclerosis. 2007;192:328-34.

IV. Paulsson JM, Moshfegh A, Dadfar E, Held C, Jacobson SH, Lundahl J.
Altered gene expression in *in vivo* extravasated neutrophils in patients with coronary artery disease.
Manuscript

CONTENTS

ABBREVIATIONS

ACE	Angiotensin converting enzyme
acLDL	Acetylated low density lipoprotein
BAL	Bronchoalveolar lavage
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CGD	Chronic granulomatous disease
CR	Complement receptor
CRP	C-reactive protein
DCFH-DA	Dichlorofluorescin diacetate
FcR	Fc receptor
fMLP	N-formylmethionyl leucyl phenylalanine
G-CSF	Granulocyte colony stimulating factor
HLA	Human leukocyte antigen
HOCL	Hypochlorous acid
HSA	Human serum albumin
ICAM	Inter cellular adhesion molecule
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
IRAK	Interleukin-1 receptor-associated kinase 1
LAD	Leukocyte adhesion deficiency
LDL	Low density lipoprotein
LFA	Lymphocyte function associated antigen
MAC	Macrophage antigen
MAPK	Mitogen activated protein kinases
MCP	Monocyte chemotactic protein
MFI	Mean fluorescent intensity
MI	Myocardial infarction
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
NADP	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa light chain enhancer of activated B cells
NGAL	Neutrophil gelatinase associated lipocalin
NS	Non-significant
oxLDL	Oxidised low density lipoprotein
PAF	Platelet activating factor
PCI	Percutaneous coronary intervention
PECAM	Platelet endothelial cell adhesion molecule
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear
PSGL	P-selectin glycoprotein ligand
ROS	Reactive oxygen species

RT-PCR	Reverse transcription polymerase chain reaction
sIL-1RII	Soluble interleukin-1 receptor type II
SOD	Superoxide dismutases
SRA	Scavenger receptor A
TNF	Tumor necrosis factors
UA	Unstable angina
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen

1 INTRODUCTION

The inflammatory process is crucial in the defense against microorganisms and in the healing of damaged tissue and ceases once the causative mediator is eliminated. However, the inflammatory reaction can become chronic and contribute to various pathological disorders. During a chronic inflammation, the processes that normally regulate defense and healing instead induce tissue destruction and fibrosis. Coronary artery disease (CAD) is characterized by a chronic inflammatory process which leads to formation of atherosclerotic plaques in the blood vessels that provide the heart with oxygen. This thesis has focused on the initial stages of an inflammatory reaction in patients with CAD and healthy subjects.

1.1 GENERAL ASPECTS OF INFLAMMATION

The inflammatory process is defined by five characteristic signs that describe the functional alterations that occur in the tissue: *calor* (heat), *dolor* (pain), *rubor* (redness), *tumor* (swelling) and *function laesa* (loss of function). Alterations in the blood vessels in proximity to the injury, such as vascular dilatation and increased permeability, induce an increased blood flow which causes redness and heat. The increased permeability enables plasma and leukocytes to accumulate at the site of injury which causes swelling. Local pain is mediated by inflammatory components that increase the sensitivity of peripheral pain receptors. Pain is a danger signal for the host and contributes to the loss of function. Additional changes include an increased expression of adhesion molecules on activated endothelial cells, which mediates leukocyte extravasation, and clotting of small blood vessels in proximity to the injury, which enclose the inflammatory location.

The establishment of an inflammatory reaction is mediated by activation of tissue resident cells, such as mast cells and macrophages that release mediators which affect the local blood vessels. The first reaction during inflammation is the innate response; it acts within minutes without adaptation. The cellular components of the innate immunity include phagocytic cells, such as neutrophils and monocytes/macrophages. The phagocytic cells circulate in blood and extravasate into injured or infected tissue through a gradient of local inflammatory mediators. At the local inflammatory site they bind to microbes and internalize them in a process called phagocytosis. The internalized microbe is then killed by fusion with granules containing microbicidal proteins and by reactive oxygen species (ROS) that are generated by a process named oxidative burst. The innate response is crucial and defects in this system can cause severe immunodeficiency.

The first line of defense is not always enough to eliminate infectious microorganisms. A second line of defense has therefore evolved which provide a more fine tuned response. The adaptive response is fully activated after four to seven days and includes leukocytes with receptors that specifically recognize the foreign antigen. Hence, a versatile reaction to the specific antigen is induced by the adaptive response. The onset of adaptive immunity is coordinated by the innate responses and depends on innate recognition of the pathogen. The cellular components of adaptive immunity include

antigen presenting cells such as dendritic cells, macrophages and B-lymphocytes and effector cells such as B- and T- lymphocytes. Antigen presenting cells process the foreign antigen and present it to the effector cells that subsequently become activated. B-lymphocytes account for the humoral immune response that generates antibodies and T-lymphocytes account for cell mediated immunity and provide signals that activate B-lymphocytes. In addition, adaptive immunity generates an immunological memory that reinforces the onset and magnitude of the defense at re-infections with the same pathogen.

Leukocytes differentiate from pluripotent stem cells in the bone marrow into two main cell lines. The myeloid lineage gives rise to monocytes, mast cells and granulocytes and the lymphoid lineage gives rise to lymphocytes. Dendritic cells can mature from both types of precursors. Acute inflammatory reactions are associated with neutrophils and monocytes/macrophages. A chronic inflammation on the other hand is mainly driven by monocytes/macrophages and lymphocytes.

1.2 NEUTROPHILS

Neutrophils, together with eosinophils and basophils, belong to a group of leukocytes designated as granulocytes, since they contain intracellular granules. Neutrophils are also called polymononuclear (PMN) cells due to their segmented nucleus that is divided into several lobes.

After approximately five days of proliferation and final maturation, mature neutrophils are released from the bone marrow to the peripheral blood [Bainton 1999]. Neutrophil trafficking from bone marrow is regulated by granulocyte colony-stimulating factor (G-CSF) that has also been endowed to reduce neutrophil apoptosis at local inflammatory sites [Gregory *et al* 2007, Semerad *et al* 2002]. Neutrophils comprise approximately 50-70% of the leukocytes in peripheral blood and circulate for about ten hours after which they die. They patrol the blood vessels for inflammatory signals and cease to circulate and migrate into inflamed tissues. The extravasation is regulated by chemokines such as CXCL8 (IL-8). The lifespan of neutrophils during inflammation is enhanced due to expression of survival signals [Altznauer *et al* 2004]. However, after approximately three days, the neutrophils undergo apoptosis and are cleared from the inflammatory site by macrophage mediated phagocytosis. Clearance may be enhanced by the release of lysophosphatidylcholine from apoptotic cells that augments the extravasation of monocytes [Lauber *et al* 2003]. By phagocytosis of apoptotic neutrophils, the neutrophil bactericidal proteins are transferred to the macrophages which enhance the macrophage mediated defense against intracellular pathogens [Tan *et al* 2006]. Clearance of redundant cells by apoptosis and macrophage phagocytosis is a prerequisite for resolution of inflammation. Pus, which is formed during some bacterial infections, is mainly composed of neutrophils and neutrophils' remnants.

Neutrophils are the first cells to extravasate into inflammatory sites. During the process of extravasation they are transformed to activated tissue dwelling defenders. A key factor in this transformation is the fine tuned sequenced release of granules and secretory vesicles. Once activated, neutrophils phagocytose foreign antigen and exert

their bactericidal actions through production of ROS and release of toxic granule components. Interaction with the target antigen can be mediated by sugar residents that bind to lectins on the bacterial surface [Ofek and Sharon 1988] or via receptors that bind to opsonised particles. Opsonisation can be mediated by antibodies, usually IgG, or components of the complement system, usually C3b, C3bi and C4b, which engage Fc receptors (FcR) and complement receptors (CR), respectively. Phagocytosis of the pathogen is controlled by receptor mediated signaling that results in rearrangement of the cytoskeleton [Strzelecka *et al* 1997]. Complement receptors that mediate neutrophil phagocytosis include CR1 and CR3 [Greenberg 1999]. CR3 is also known as the adhesion molecule CD11b/CD18 and have therefore dual roles. FcγRIII, also called CD16, is highly expressed on neutrophils and can be used to discriminate neutrophils from other leukocytes.

During differentiation in the bone marrow, neutrophils are armed with effector molecules. These are packed within cytoplasmic stores, sequentially formed during differentiation in the following order: azurophil granules (primary), specific granules (secondary), gelatinase granules (tertiary) and secretory vesicles [Faurschou and Borregaard 2003]. The content of the granules is regulated by the sequential timing of the molecular biosynthesis during the time of granule development. The granules contain bactericidal proteins such as cationic defensins and the bactericidal/ permeability increasing protein, which disrupt the negatively charged bacterial cell wall. Granules also contain serin proteases and matrixmetalloproteinases (MMPs) with bactericidal and proteolytic functions. In addition, the granule membranes contain receptors for pattern recognition and phagocytosis, adhesion molecules and components required for the oxidative burst. Simplified, azurophil and specific granules contain bactericidal and proteolytic proteins that are released intracellularly by fusion with phagocytic vesicles [Joiner *et al* 1989]. However, the specific granules can also be released extracellularly [Sengelov *et al* 1995]. The release of bactericidal and matrix degrading proteins from gelatinase granules occur mainly extracellularly [Mollinedo *et al* 1997]. Secretory vesicles are easily translocated to the plasma membrane [Borregaard *et al* 1987]. They contain plasma proteins and membrane receptors associated with adhesion and pattern recognition, such as CR1, CR3 and CD16, and they

It was long thought that fully differentiated neutrophils were transcriptionally silent. However, a recent publication suggests that activation of biosynthesis occurs following extravasation [Theilgaard-Monch *et al* 2006]. This transcriptional activation includes induction of cytokines and chemokines [Theilgaard-Monch *et al* 2004, Coldren *et al* 2006]. Hence, neutrophils may tune the subsequent inflammatory response. Theilgaard-Monch *et al* demonstrated that extravasated neutrophils had little transcriptional activity in genes categorized for bactericidal defense, extravasation or changes in cell structure [Theilgaard-Monch *et al* 2004]. In contrast, a substantial transcriptional activation occurred in genes regulating apoptosis, leukocyte recruitment, proliferation, angiogenesis and modulation of extracellular matrix. The transcriptional activator, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), is a key factor for gene expression following stimulation with inflammatory agents such as interleukin 1 (IL-1) in human neutrophils [Malcolm *et al* 2003, McDonald *et al* 1997, McDonald and Cassatella 1997].

Neutrophils can regulate the subsequent extravasation of other leukocytes by the production and release of chemotactic substances. This has been demonstrated in neutropenic mice that had a reduced extravasation of monocytes that were restored by administration of secretion products from neutrophils [Soehnlein *et al* 2008]. Neutrophils modulate the local inflammatory milieu by synthesis of chemokines [Scapini *et al* 2000] and by protease mediated modification of already present chemokines [Padrines *et al* 1994, Berahovich *et al* 2005]. In addition, some granule components such as LL-37 are chemotactic for neutrophils and mononuclear cells [De *et al* 2000].

In recent years it has also been demonstrated that neutrophils influence the adaptive immune response through transport and presentation of antigens in lymphoid tissue [Appelberg 2007]. It is becoming evident that neutrophils, that are the major leukocyte population in peripheral circulation, have far reaching effect beyond the role as a phagocyte and may set the tune for the subsequent events once the inflammatory process has commenced.

1.3 MONOCYTES AND MACROPHAGES

Monocytes comprise 5-10% of the peripheral circulating leukocytes. They develop in the bone marrow, circulate in peripheral blood and migrate into tissue during steady state and inflammation [van Furth 1985]. Extravasated monocytes differentiate into either macrophages or dendritic cells, and this transition is a dynamic process. These cells are therefore commonly termed monocyte derived. Monocyte derived cells are particularly interesting since they mediate innate responses and are a prerequisite for initiation of adaptive immunity. In addition, monocyte derived cells have a longer survival than neutrophils.

Approximately 90% of the circulating monocytes have a high expression of CD14 and lack expression of CD16; these cells are therefore termed $CD14^+CD16^-$. In contrast, the $CD14^+CD16^+$ monocytes account for approximately 10% of the circulating monocytes [Passlick *et al* 1989]. The $CD14^+CD16^+$ monocytes are proposed to be more differentiated than the $CD16^-$ monocytes [Ziegler-Heitbrock *et al* 1993, Ancuta *et al* 2000] and it might be that the $CD16^+$ monocytes more readily migrate to inflammatory sites. Recent data indicates that $CD16^+$ and $CD16^-$ monocytes may give rise to different macrophage and dendritic cell progeny [Ancuta *et al* 2009]. However, there is still much to unravel about the subtypes of monocytes. A role for the $CD14^+CD16^+$ monocytes in patients with CAD and on hemodialysis has been put forward [Schlitt *et al* 2004, Nockher and Scherberich 1998]. The $CD14^+CD16^+$ monocytes have an increased expression of pro-inflammatory cytokines, increased antigen presentation and reduced phagocytosis and oxidative burst [Ziegler-Heitbrock 2007, Ziegler-Heitbrock 1996].

Macrophages are tissue resident cells, part of the stromal network and associate with the endothelium and epithelium. They are rapidly activated in case of tissue injury or bacterial encounter, and provide activation signals to the endothelium. Macrophages are

constitutively recruited from circulating blood monocytes [Gordon 1999]. Early monocyte differentiation is influenced by adhesive events during extravasation and by the local inflammatory milieu [Wesley *et al* 1998, Sudhakaran *et al* 2007, Wang *et al* 2001, Chomarat *et al* 2000]. Resident macrophages are phenotypically diverse and differentiate according to their local environment. They can be categorized into two main subtypes, classically activated M1 and alternatively activated M2 [Martinez *et al* 2006]. However, these subtypes do not reflect the different roles of macrophages in tissue and the M2 group is further divided into subgroups. In a recent paper, Moser et al [Mosser and Edwards 2008] divides macrophages into three categories, classically activated, wound healing and regulatory and suggests that the phenotype of macrophages is highly plastic and that all kinds of transitional stages can occur. Monocytes are transiently modified by the cytokine environment which is influenced by the local influx of leukocytes. The function of the $CD14^{+}CD16^{-}$ and the $CD14^{+}CD16^{+}$ monocytes and to what degree these replenish macrophage and dendritic cell populations is not fully understood and needs further studies to be delineated. During differentiation, monocyte derived cells acquire an increased ability for antigen presentation, which is seen as an up-regulation of human leukocyte antigen (HLA) and the co-stimulatory molecules CD80 and CD86 [Santin *et al* 1999, Laupeze *et al* 1999].

Compared to neutrophils, monocyte derived cells have a slower onset of extravasation during the initial stages of inflammation. Like neutrophils, they participate in phagocytosis and in oxidative burst [Dale *et al* 2008]. Macrophage phagocytosis induces clearance of apoptotic cell and microorganisms and provides the macrophage with antigens to be presented. Hence, macrophages are involved in tissue homeostasis as well as in innate and adaptive immunity. Macrophage phagocytosis is mediated by carbohydrates, FcR and CR that bind antigens in a similar manner as in neutrophils. Macrophages also express scavenger receptors that bind to polyanionic ligands and mediate clearance of apoptotic cells and cellular debris. The class B scavenger receptor, CD36, is utilized in clearance of apoptotic cells [Fadok *et al* 1998]. In addition, macrophages express toll like receptors that are involved in pattern recognition and signal the presence of virus and bacteria.

Following stimulation, macrophages release IL-1β and tumor necrosis factor α (TNF-α), both with profound effects on endothelial cell activation [Furie and Mc Hugh 1989]. Macrophages also release IL-6 and IL-6, IL-1β and TNF-α induce an acute phase response in the liver. An indicator of the acute phase is the increase in systemic C-reactive protein (CRP). Macrophages also produce chemokines, such as IL-8, macrophage inflammatory proteins 1α and 1β (MIP-1α and 1β), monocyte chemotactic protein-1 (MCP-1) and RANTES that induce further leukocyte extravasation.

1.4 CONSECUTIVE STEPS IN THE EXTRAVASATION PROCESS

Extravasation occurs primarily at post capillary venules, which are small vessels with a thin layer of smooth muscle cells. Leukocyte extravasation *in vivo* is divided into several consecutive steps: rolling, activation, firm adhesion and transmigration. In recent years these steps have been refined. Ley *et al* presented a model that included

capture, rolling, slow rolling, arrest, adhesion strengthening, crawling and paracellular or transcellular transmigration [Ley *et al* 2007].

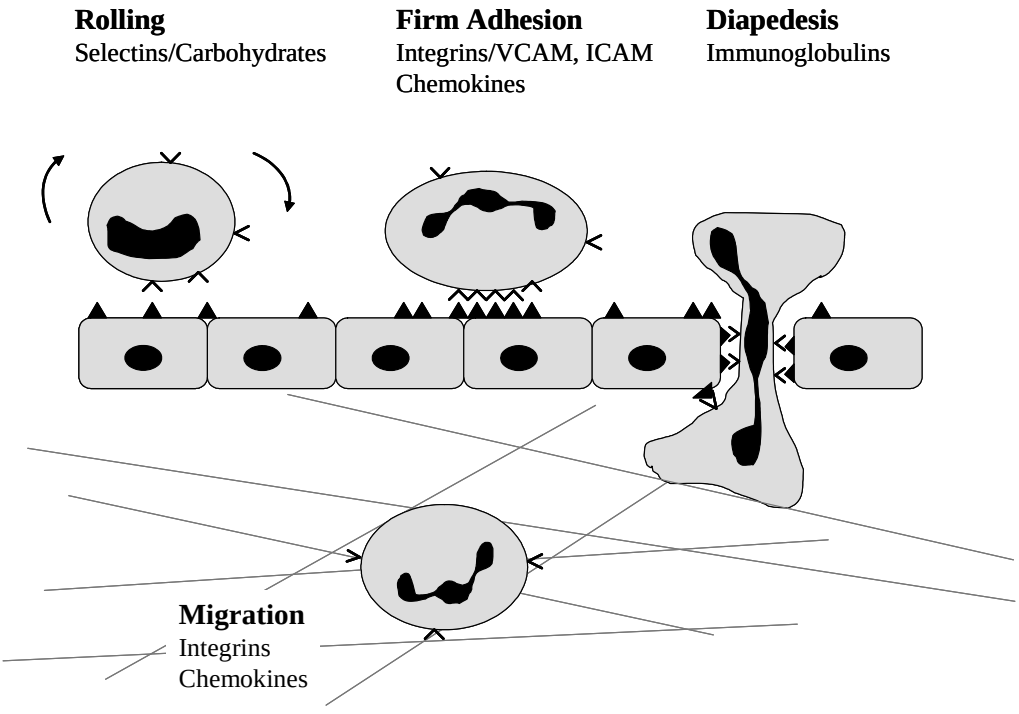

Figure 1. The consecutive steps of extravasation.

1.4.1 Endothelial activation

Activation of the endothelium occurs in two steps during an inflammatory response [Pober and Sessa 2007]. The initial activation is mediated by G-protein coupled receptors such as the histamine receptor. The outcome of this activation is opening of endothelial gap junctions, exocytosis of P-selectin and production of platelet activating factor (PAF). The subsequent activation is mediated by TNFα and IL-1 which induce the transcription factors NFκB and activator protein 1 that regulate the production of adhesion molecules and chemokines. In addition, migrating leukocytes activates the endothelium and induces opening of endothelial gaps [Hixenbaugh 1997].

1.4.2 Selectin mediated steps

The initial rolling contact with the endothelium is mediated by selectins which contain lectin domains that bind to carbohydrates. L-selectin (CD62L, LAM-1) is expressed on most circulating leukocytes, whereas E-selectin (CD62E, ELAM-1) and P-selectin (CD62P, LECAM-3) are expressed on endothelial cells during inflammation. Following inflammatory activation, P-selectin is mobilized from intracellular stores [McEver *et al* 1989] and the production of E-selectin is initiated in endothelial cells [Bevilacqua *et al* 1987]. The ligand for P-selectin, P-selectin glycoprotein ligand-1

(PSGL-1), mediates leukocyte rolling. PSGL-1 can also bind to L-selectin and thereby mediate secondary capture by which rolling leukocytes capture each other [Paschall and Lawrence 2008]. The interactions during rolling are affected by shear stress that modulates the strength of selectin interactions. This is one explanation for the attachment and detachment that characterize rolling [Marshall *et al* 2003].

1.4.3 Integrin mediated steps

Integrins are heterodimers consisting of one α and one β chain. The integrin family is continuously growing and there are at least 18 α chains and eight β chains that generate 24 different heterodimers sorted into different subfamilies according to their β subunit [Takada Gen *et al* 2007]. The extravasation of monocytes and neutrophils is particularly associated with β_1 and β_2 integrins. The β_1 integrins bind to components in the extracellular matrix such as laminin, collagen, fibronectin and vitronectin. The β_2 integrins can bind to matrix components, but are more involved in cell-cell contacts. Integrins mediate firm adhesion to endothelial cells. However, integrin mediated rolling during inflammatory conditions has been reported [Gaboury and Kubes 1994, Dunne *et al* 2002, Berlin *et al* 1995]. Rolling and firm adhesion by β_2 integrins might be synchronized by altering the affinity for the counter receptor on the endothelium [Salas *et al* 2004].

The β_1 integrin $\alpha_4\beta_1$ (very late antigen-4 (VLA-4)) is mainly associated with mononuclear cells. VLA-4 binds to vascular cell adhesion molecule-1 (VCAM-1) on activated endothelial cells [Chuluyan and Issekutz 1993]. VLA-4 is normally not expressed on neutrophils but can be induced by nitric oxide [Conran 2003]. Although VLA-4 may not be directly involved in neutrophil adhesion, a cross talk between $\beta_1$and β_2 integrins has been suggested [van den Berg *et al* 2001].

There are three main β_2 integrins: $\alpha_L\beta_2$ (CD11a/CD18, lymphocyte function-associated antigen 1 (LFA-1)), $\alpha_M\beta_2$ (CD11b/CD18, macrophage-1 antigen (MAC-1), CR3) and $\alpha_x\beta_2$ (CD11c/CD18, CR4). The β_2 counter-receptor on activated endothelial cells is intracellular adhesion molecule 1 (ICAM-1) [Diamond *et al* 1990, Smith *et al* 1989]. LFA-1 can also bind to ICAM-2, which is constitutively expressed on resting endothelium [de Fougerolles *et al* 1991]. Hence, ICAM-2/LFA-1 interaction can mediate leukocyte migration without inflammatory activation while ICAM-1 interactions with β_2 integrins are dependent on inflammatory activation. The CD11b/CD18 and CD11c/CD18 integrins may also bind to fibrinogen and complement fragment iC3b and have several overlapping functions.

Integrin mediated adhesion is regulated by altering the amount and affinity of the integrins. Neutrophil and monocyte activation is associated with up-regulation of CD11b/CD18 through mobilization of intracellular granules [Borregaard *et al* 1994, Miller 1987]. The high affinity conformation of integrins is induced by chemokines. The α-chain of many integrins contains a metal ion binding I-domain that can form an inactive closed conformation or an active and open conformation [Takada *et al* 2007]. In addition, the β-chain contains an I-like domain with similarities to the I-domain. Chemokines induce an inside out signaling that results in phosphorylation of the β chain and hence dissociation from the α- chain [Takada *et al* 2007]. This is dependent

on the rearrangement of the cytoskeleton which is mediated by talin that binds the cytoplasmic tail of the β chain [Ratnikov *et al* 2005]. The high affinity form is hence associated with separation of the α and β chains as well as elongation of the integrin conformation.

Leukocyte arrest is associated with integrin clustering in the cell membrane and this induces an outside-in signaling that modulates cellular function. Functional alterations include rearrangements of the cytoskeleton, phagocytosis, degranulation, oxidative burst, cytokine production and regulation of apoptosis [Zarbock and Ley 2008, Anderson *et al* 1986, Shappell *et al* 1990, Graham *et al* 1989, Couturier *et al* 1990, Coxon *et al* 1996]. The outside- in signaling is mediated by changes in the integrin cytoplasmic region and activation of Src kinases [Abram and Lowell 2009].

The importance of integrins in innate immunity is indicated by the leukocyte adhesion deficiency (LAD) syndrome that is associated with defects in the β_2 chain [Kishimoto *et al* 1989]. The symptoms of LAD are associated with reduced adherence dependent functions such as chemotaxis, phagocytosis and oxidative burst.

1.4.4 Diapedesis

Preceding transmigration, the leukocytes crawl on the endothelium seeking the right location to penetrate. Most migration occurs between endothelial cells through cell junctions and involves adhesion molecules in the immunoglobulin superfamily such as platelet endothelial cell adhesion molecule 1 (PECAM-1) and ICAM, as well as CD99 [Muller *et al* 1993, Ley *et al* 2007]. A transcellular route engaging ICAM-1 has also been defined [Millan *et al* 2006].

1.4.5 Extracellular matrix and the basement membrane

The extracellular matrix comprises the connective tissue. The basal membrane, which is a sheath of matrix underneath the endothelium or epithelium, is mainly composed of collagen IV and laminin. Both monocytes and neutrophils have been shown to migrate over the basement membrane preferentially at locations with thinner protein content. The extracellular release of proteases during extravasation could indicate that leukocyte migration is associated with matrix degradation [Sengelov *et al* 1995], however, this is not fully known. Neutrophils, in contrast to monocytes, have recently been shown to mediate matrix degradation during *in vivo* extravasation in a mouse model [Voisin *et al* 2009]. Adhesive contact to the extracellular matrix is mediated by β_1 integrins and migration is directed towards gradients of chemokines bound to heparan sulfate proteoglycans [Celie *et al* 2009].

1.5 CHEMOATTRACTANTS

Chemoattractants induce extravasation and regulate leukocyte functions. Chemoattractants can be products from bacteria, fragments of complement, phospholipid metabolites and chemokines. Bioactive lipids with chemotactic activity include leukotrienes and PAF, these are not further discussed.

1.5.1 fMLP

Formylated peptides released from bacteria and disrupted mitochondria are strong inducers of leukocyte extravasation [Schiffmann *et al* 1975]. The bacterial tripeptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) is commonly used to study leukocyte chemotaxis and activation. fMLP bind to two receptors with different affinities [Fu *et al* 2006, Ye *et al* 1992]. The receptors are located in the plasma membrane and in intracellular granules that are mobilized during inflammation [Sengelov *et al* 1994]. The receptors are coupled to G-proteins and ligand binding initiates several pathways, among them activation of phosphoinositide 3- kinase, phospholipase C (PLC), protein kinase C (PKC), mitogen activated protein kinases (MAPK), Ca^{2+} mobilization, small GTPases, NFκB and others [Selvatici *et al* 2006, Panaro *et al* 2006, Browning *et al* 1997]. Activation by fMLP induces many different cellular functions including migration, degranulation, oxidative burst and cytokine production.

1.5.2 C5a

The complement system is activated by microbial encounter. However, a cross talk between complement and coagulation has been reported [Huber-Lang *et al* 2006]. In addition, phagocytic cells have been shown to generate C5a independently of the traditional mechanisms for complement activation [Huber-Lang *et al* 2002]. The complement cascade triggers consecutive proteolytic cleavages that ultimately produce biologically active peptides with chemotactic, opsonising or cytolytic functions [Fernandez *et al* 1978]. C5a is one of the most important products and induces various signaling pathways by binding to G-protein coupled receptors [Monk *et al* 2007].

1.5.3 Chemokines

Chemokines contain repeated cysteins connected by disulfide bonds and the position of the first two cysteins divide the chemokines into different groups. CC-chemokines have adjacent cysteins while CXC and CX_3C chemokines have cysteins separated by one or three amino acids, respectively. Chemokines with a function during inflammation are induced by IL-1, TNF, microbial encounter or phagocytosis [Thorburn *et al* 2009, Hachicha *et al* 1998]. In addition, endothelial cells produce chemokines following leukocyte interaction [Lukacs *et al* 1995]. Chemokines are produced by resident cells such as macrophages and mast cells, endothelial cells, epithelial cells, fibroblasts and by extravasated leukocytes. Chemokines bind to sugar residents on endothelial cells and in the extracellular matrix and are thereby gradually captured at the site of inflammation [Johnson *et al* 2005]. The importance of a gradient has been demonstrated by intravenous administration of CXCL8 (IL-8) that blocked leukocyte chemotaxis during a dermal inflammation [Hechtman *et al* 1991].

1.5.4 Regulation of chemokine activity

There are many different chemokines with overlapping functions and they can be classified as inflammatory, homeostatic or with dual-functions [Moser *et al* 2004]. In addition, many chemokines can bind to several overlapping receptors. Overlapping receptors with different affinity have been reported in for the CXC chemokines [Loetscher *et al* 1994]. Chemokines can also be modified by gelatinase that has been shown to increase the activity of IL-8 by truncation. The increased activity was more

pronounced for CXCR1 compared to CXCR2 [Van den Steen *et al* 2000]. CCL chemokines are also modified by truncations. Many chemokines form dimers and this might be an additional way of regulation. Dimerisation is important for suppression of adherence dependent oxidative burst by IL-8 in neutrophils [Williams *et al* 2005].

1.5.5 Chemokine signaling

Chemokines bind to heterotrimeric G-protein coupled receptors. The signaling cascades include activation of PLC, phosphoinositide 3-kinase, c-Src family of tyrosine kinases, Ca^{2+} mobilization, PKC and MAPK, among others [Thelen 2001, Thelen and Stein 2008]. Chemokines account for the inside out signaling that alters the integrin affinity, regulates migration and prime the leukocyte for degranulation and oxidative burst. Although fMLP and chemokines initiate similar signaling pathways, induction of oxidative burst in neutrophils indicate that fMLP and IL-8 may exert their functions through different mechanisms [Fu *et al* 2004]. In addition, fMLP and IL-8 activate integrins differently during chemotaxis *in vitro* [Heit *et al* 2005].

A hierarchy between different attractants exists and end target chemoattractants, such as fMLP and C5a, dominates over endogenous chemoattractants, such as IL-8 and leukotriens [Campbell *et al* 1997, Heit *et al* 2002]. The hierarchy between end target and endogenous chemoattractants is partly mediated by their different means of intracellular signaling and the engagement of different adhesion molecules [Heit *et al* 2002]. The hierarchy ensures that exogenous signals dominate over host derived signals.

Chemokine	**Category**	**Receptor**	**Receptor expression**
CCL2 (MCP-1)	Inflammatory	CCR2	Monocytes, Basophils, T-lymphocytes$^+$
CCL3 (MIP-1α)	Inflammatory	CCR1	Monocytes, Basophils, T-lymphocytes$^+$
		CCR5	Monocytes$^+$, T-lymphocytes$^+$,
CCL4 (MIP-1β)	Inflammatory		Dendritic cells
CCL20 (MIP-3α, LARC)	Dual	CCR6	T-lymphocytes$^+$, B-lymphocytes, Dendritic cells
CXCL2 (MIP-2α, GRO-β)	Inflammatory	CXCR2	Neutrophils, Monocytes
CXCL8 (IL-8)	Inflammatory		
		CXCR1	Neutrophils
CX_3CL1 (Fractalkine)	Inflammatory	CX_3CR1	Monocytes/Macrophages, T-lymphocytes$^+$

Table 1. Characteristics of chemokines that have been studied within this thesis.

1.5.6 Fractalkine

Fractalkine (CX_3CL1) is the only known member of the CX_3C family. Fractalkine is distinguished from other chemokines by the presence of a transmembrane domain by which it binds to endothelial cells. Proteolysis of the membrane bound form generates a soluble form with chemotactic activity. By its different means of expression, fractalkine mediates selectin and integrin independent adhesion as well as chemotaxis [Umehara *et al* 2004, Imai *et al* 1997]. Fractalkine is particularly important for the extravasation of the $CD14^+CD16^+$ monocytes [Ancuta *et al* 2003].

1.6 LEUKOCYTE PRIMING, ACTIVATION AND DESENSITIZATION

Leukocyte responses can be amplified by priming and down-regulated by desensitization. This way of regulating the inflammatory response ensures a rapid beginning and a controlled termination.

1.6.1 Priming and activation

Chemoattractants induce firm adhesion and prime leukocytes for further cytotoxic responses such as oxidative burst. IL-8 priming of fMLP induced oxidative burst includes a sequential gathering of the reduced form of nicotinamide adenine dinucleotide phosphate (NADP), the NADPH oxidase, in neutrophils [Guichard *et al* 2005]. Priming by C5a mediates partially different pathways for activation of oxidative burst by phorbol 12-myristate 13-acetate (PMA) or E-coli [Wrann *et al* 2007]. In addition, inflammatory cytokines and β_2 integrin cross linking mediates priming of fMLP induced oxidative burst and expression of adhesion molecules [Condliffe *et al* 1996, Wittmann *et al* 2004, Elbim *et al* 1994, Liles *et al* 1995]. Priming is also associated with inflammatory conditions. Neutrophil priming by immunoglobulin aggregates in synovial fluid has been detected in patients with rheumatoid arthritis [Robinson *et al* 1992]. The mechanism of priming is not fully understood and involves several levels of regulation that may differ between different agonists. Potential mechanisms include G-protein mediated events and their downstream signaling pathways as well as tyrosine phosphorylations. Different means of priming has been reviewed by Condliffe *et al* [1998]. Furthermore, reversible priming has been suggested for PAF which favors the idea of a balance between priming and de-priming in the regulation of neutrophil activity [Kitchen 1996].

1.6.2 Desensitization

G-protein coupled receptors are phosphorylated and internalized following ligand binding and this response mediates a rapid homologous desensitization [Uhing and Snyderman 1999]. Regained responsiveness is regulated by a balance between receptor degradation, de-phosphorylation and hence re-expression as well as synthesis of new receptors. Homologous desensitization has been reported for the receptors of fMLP, C5a, IL-8, leukotrienes, PAF and MCP-1 [Tomhave *et al* 1994, Franci *et al* 1996]. Heterologous desensitization that occurs between different G-protein coupled receptors is mediated at the level of second messengers, such as PLC and does not involve ligand binding [Uhing and Snyderman 1999]. Heterologous desensitization is dependent on the type of G-protein the respective receptor utilizes. Cross phosphorylation has been

detected between the fMLP, C5a and IL-8 receptors. Desensitization of the C5a and IL-8 receptors is likely to involve PKC [Richardson *et al* 1995, Tomhave *et al* 1994]. The fMLP receptor on the other hand has no domains for PKC phosphorylation and desensitization by IL-8 or C5a is therefore likely to involve other mechanisms related to PLC [Richardson *et al* 1995].

1.7 THE OXIDATIVE BURST, PRODUCTION OF ROS

1.7.1 The NADPH oxidase

Phagocytic cells synthesize toxic oxygen metabolites as part of their effector functions. The first reaction is mediated by the NADPH oxidase that catalyses the reduction of O_2 into the superoxide anion (O_2^-). The NADPH oxidase is composed of membrane and cytosolic components that translocate and assemble in the plasma or granule membranes following activation. Phosphorylation is essential for subunit translocation and partial assembly is seen following priming [Sheppard *et al* 2005]. However, complete assembly and production of O_2^- requires full activation. The membrane bound part of the NADPH complex, cytochrome b_{558}, is located to 80-90% in specific granules. These are translocated to the plasma and phagosomal membranes following phagocytosis or stimulation [Borregaard *et al* 1983, Jesaitis *et al* 1990]. Chemoattractants mainly induce NADPH oxidase activity in the plasma membrane while phagocytosis induces activity in intracellular granules [Karlsson and Dahlgren 2002]. Production of O_2^- has also been demonstrated following integrin engagement [Berton *et al* 1992, Yan and Novak 1999].

The importance of the NADPH oxidase system is indicated in patients with chronic granulomatous disease (CGD). These patients have a defect in superoxide production and suffer from recurrent bacterial and fungal infections [Holmes *et al* 1967, Quie *et al* 1967].

1.7.2 Formation of hydrogen peroxide

The superoxide anion is dismutated into hydrogen peroxide (H_2O_2) and this reaction can be either spontaneous or catalyzed by the superoxide dismutase (SOD) [Klebanoff 1999]. The intracellular production of H_2O_2 can be measured by flow cytometry using dichlorofluorescin diacetate (DCFH-DA) labeled cells [Bass *et al* 1983]. The DCFH-DA molecule diffuses over the cell membrane and accumulates intracellularly following desacetylation. The desacetylation product, DCFH, is then oxidized in the presence of H_2O_2 and the oxidized product is fluorescent. The DCFH-DA system enables a quantitative assessment of the intracellular H_2O_2 production. PMA, which is a direct activator of PKC and commonly used to study the production of oxygen radicals, induces both intra- and extracellular production of H_2O_2 in approximately similar amounts [Lundqvist *et al* 1996].

There are several scavenger systems that degrade H_2O_2. These include the catalase and the glutathione peroxidase systems, and they protect the host from the toxic effects of H_2O_2 [Voetman and Roos 1980, Roos *et al* 1979]. Extracellular release of ROS accounts for tissue destruction and fibrosis associated with chronic inflammation.

1.7.3 Reactions between H_2O_2 and myeloperoxidase

Myeloperoxidase (MPO) is stored in the azurophil granules [Bainton *et al* 1971] and catalyses further modification of H_2O_2 into more potent radicals. The main reaction occurs between MPO, H_2O_2 and chlorine and generates hypochlorous acid (HOCL). The MPO/H_2O_2 system can also mediate production of reactive nitrogen intermediates, tyrosyl radicals and halogenation by chloride as well as other halides [Klebanoff 2005]. The products of this system are toxic to a broad spectrum of infectious agent and in addition, they inactivate bacterial toxins [Klebanoff 1999].

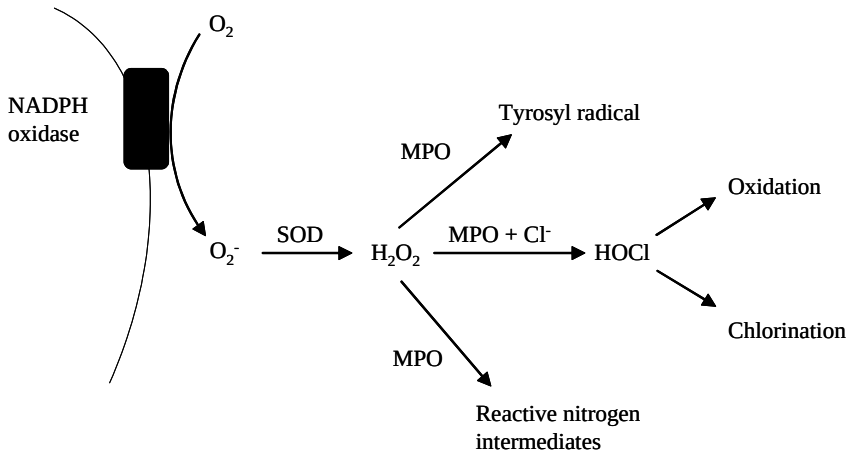

Figure 2. Production of reactive oxygen species.

1.8 THE SKIN CHAMBER METHOD

Most studies of human leukocytes utilize cells from peripheral blood or cells collected from local inflammatory sites such as bronchoalveolar lavage (BAL) or synovial fluids. In order to study *in vivo* activated leukocytes from patients with atherosclerosis, we have used a skin chamber method. This method enables the study of leukocytes that have extravasated to an inflammatory site *in vivo* [Follin and Dahlgren 2007]. Skin blisters are induced by suction and gentle heating and this separates epidermis from the underlying basal membrane and dermis [Kiistala and Mustakallio 1964, 1967]. The blister roofs that contain epidermis are removed and plastic chambers are mounted over the exposed wounds. The dermal papillae capillaries are then exposed to an inflammatory stimulation. The inflammatory reaction can be stimulated by autologous serum [Kuhns *et al* 1992], zymosan activated autologous serum [Forsgren and Scheja 1985], isotonic salt buffer [Perillie and Finch 1964] or allergens [Nopp *et al* 2000]. In the present thesis, we have used autologous serum to induce an intense inflammatory reaction and PBS to induce an intermediate inflammatory reaction.

Leukocyte extravasation into the skin chamber occurs at dermal capillaries and venules and includes interaction with endothelial cells and the extracellular matrix in the basal

membrane. The detached leukocytes are dominated by neutrophils witch constitute over 90 % of the extravasated cells after 12-24 hours [Kuhns *et al* 1992, Koivuranta-Vaara 1985, Forsgren and Scheja 1985]. The extravasation of monocytes is slower and monocytes constitute 15% of the exudated cells after 10 hours.

1.8.1 The inflammatory milieu

Studies with heat inactivated and zymosan activated autologous serum indicate that mediators produced by complement activation are crucial in the inflammatory milieu [Kuhns *et al* 1992, Forsgren and Scheja 1985, Scheja and Forsgren 1985]. A major component during the early inflammatory reaction is C5a which is accompanied by IL-8 after a few hours [Follin *et al* 1991]. Additional inflammatory mediators in the skin chamber include interferon-γ, leukotriene-B4, IL-6, IL-1β, TNFα and GM-CSF [Kuhns *et al* 1992]. The inflammatory milieu in the skin chamber is orchestrated by extravasated leukocytes and dermal mast cells and could be further influenced by fibroblasts and tissue macrophages in the deeper layers of the skin.

1.8.2 Studies of leukocyte function

Extravasated cells can be both primed and desensitized. Desensitization of exudated cells has been noted for C5a and IL-8, the major inflammatory mediators in the chamber exudate [Follin *et al* 1991]. Priming has been noted for fMLP and relates to an increased expression of fMLP receptors due to granule mobilization. An increased expression of fMLP receptors and CD11b/CD18, as well as augmentation of fMLP induced oxidative burst, has been reported for exudated neutrophils [Zimmerli *et al* 1986]. The absence of priming towards PMA, a stimuli that bypasses receptor activation, indicated a receptor specific response. During extravasation secretory vesicles and granules are mobilized [Wright and Gallin 1979]. The order of mobilization follows in the reverse order of the formation. Extravasation to the skin chamber mobilizes 100% of the secretory vesicles, 40% of the gelatinase granules, 20% of the specific granules and 10% of the azurophilic granules [Sengelov *et al* 1995]. Additional alterations in extravasated leukocytes include increased expression of VLA-2 and CD11b/CD18 [Werr *et al* 2000, Sengelov *et al* 1995] and shedding of L-selectin [Kuhns *et al* 1995]. Extravasated neutrophils are also transcriptionally activated [Thielgaard-Monch *et al* 2004].

The past decade, the skin chamber model has been applied to evaluate basic leukocyte function following extravasation in healthy individuals [Thielgaard-Monch *et al* 2004], patients with allergy [Nopp *et al* 2000] and patients with renal disease [Dadfar *et al* 2004]. For the first time, we have applied the skin blister model to evaluate functionally activated leukocytes in patients with CAD.

1.9 CORONARY ARTERY DISEASE

The coronary arteries supply the myocardium with nutrients and oxygen. Development of atherosclerotic plaques in the wall of these vessels gives raise to CAD and clinical manifestations such as angina pectoris and myocardial infarction (MI). CAD is the most common cause of acute hospitalization in Sweden and MI is the most frequent

individual cause of death. The incidence of CAD is highly related to age and women have a later onset (8-10 years) than men. Individual risk factors for CAD include smoking, hypertension, obesity, hypercholesterolemia, diabetes mellitus and inherited factors. Arteries with occlusions or stenoses can be treated by percutaneous coronary intervention (PCI) where the vessel lumen is widened by a balloon. This procedure is often associated with the insertion of a metal network called stent to prevent the risk of restenosis. Affected arteries may also be surgically bypassed by a coronary artery bypass graft (CABG). Pharmacological treatments include lipid lowering therapy with statins, antithrombotic therapy (aspirin and clopidogrel), β-receptor blockers and the use of angiotensin-converting enzyme (ACE) inhibitors.

Atherosclerotic lesions within the intima of the vessel wall build up during decades. Small inclusions of fat, called fatty streaks, can be detected in children and with increasing age these inclusions may cause pathological atherosclerotic plaques. Atherosclerosis is associated with an inflammatory reaction in the plaque, but the initial trigger is not fully known. A combination of multiple factors that induce local stress to the coronary arteries such as an altered blood flow, smoking and infections might contribute. Modification of the cholesterol molecule low density lipoprotein (LDL) into oxidized LDL (oxLDL) is crucial for plaque development and contributes to inflammation. Macrophages within the plaque bind to oxLDL and are transformed into lipid loaded foam cells. The coronary arteries have a dense layer of smooth muscle and in advanced stages of atherosclerosis these become activated. The advanced plaque is composed of a lipid core surrounded by a fibrous cap of smooth muscle cells and extracellular matrix. The fibrous cap is protective and breakdown of the cap causes tissue factor in the lesion to leak into the blood, causing the formation of a thrombus. The blockage of coronary arteries by a thrombus initiates myocardial infarction. The atherosclerotic plaque is composed of monocyte derived foam cells, smooth muscle cells and some T-lymphocytes. Occasionally, other cell types such as neutrophils are found.

1.9.1 Monocytes in CAD

Monocyte derived cells are the dominant cell type in the lipid core [Jonasson *et al* 1986, Bonanno *et al* 2000]. The monocytes are mainly recruited from the circulation [Lessner *et al* 2002] by expression of chemokines such as CCL2 in the atherosclerotic plaques [Nelken *et al* 1991]. The number of circulating $CD14^+CD16^+$ monocytes is slightly increased in CAD compared to in healthy controls [Schlitt *et al* 2004] and these cells predict the incidence of cardiovascular events in patients on dialysis [Heine *et al* 2008]. The $CD14^+CD16^+$ monocytes produce substantial amounts of TNFα [Belge *et al* 2002] and the number of circulating $CD14^+CD16^+$ monocytes associate with the concentration of TNFα in serum [Schlitt *et al* 2004]. Fractalkine is expressed on human atherosclerotic endothelium which indicates a rout of extravasation for the $CD14^+CD16^+$ monocytes [Volger *et al* 2007, Yano *et al* 2007]. Knock out studies in mice indicate that fractalkine is important in CAD due to the extravasation of monocyte derived cells which contribute to plaque growth [Lesnik *et al* 2003]. The expression of fractalkine on smooth muscle cells is up-regulated by oxLDL [Barlic *et al* 2007] and CX_3CR1 signaling may promote the survival of monocytes in the plaque [Landsman *et al* 2009].

1.9.1.1 Scavenger receptors and modified cholesterol

Following extravasation, monocytes differentiate into macrophages which are transformed into lipid loaded foam cells. A prerequisite in this process is the binding of modified cholesterol to scavenger receptors. The type B scavenger receptor, CD36, is up-regulated during macrophage differentiation [Huh *et al* 1996] and can be detected on foam cells in human atherosclerotic plaques [Nakata *et al* 1999]. Many scavenger receptors have the capacity to bind to modified lipids, but CD36 is the main receptor for oxLDL [Endemann *et al* 1993]. Ligands for CD36 can be generated by the MPO/H_2O_2/nitrite system [Podrez *et al* 2000]. CD36 has overlapping affinities with other scavenger receptors, especially scavenger receptor class A1 (SR-A1). Both bind to oxLDL and acetylated LDL (acLDL), but CD36 has highest affinity to oxLDL, while SRA has high affinity to acLDL [Kunjathoor *et al* 2002]. Following *in vitro* incubation with modified LDL, smooth muscle cells and endothelial cells increase their expression of CCL2 [Cushing *et al* 1990] and macrophages increase their expression of CD36 [Han *et al* 1997]. This implies that LDL mediates several pro-inflammatory mechanisms that are associated with atherosclerosis. In addition, oxLDL induced signaling from CD36 inhibits macrophage migration and could mediate macrophage trapping [Park *et al* 2009]. The $CD14^+CD16^+$ monocytes have a lower gene expression of SR-A1 and a similar expression of CD36 as the $CD14^+CD16^-$ monocytes [Draude *et al* 1999]. However, the binding of modified LDL to $CD14^+CD16^+$ monocytes has not been fully elucidated [Draude *et al* 1999, Mosig *et al* 2009].

1.9.1.2 Antigen presentation

Extravasated monocytes may differentiate into antigen presenting cells, indicated by an increased expression of HLA and co-stimulatory molecules. The co-stimulatory molecules CD80 and CD86 have been detected on macrophages in human atherosclerotic lesions [deBoer *et al* 1997]. *In vitro* differentiated dendritic cells from patients with CAD have an increased expression of co-stimulatory molecules compared to cells differentiated from healthy subjects [Dopheide *et al* 2007]. Knock out studies in mice indicate that CD86 and CD80 are important for activation of T- lymphocytes and development of atherosclerotic lesions [Buono *et al* 2004]. The presence of activated T-lymphocytes in human plaques further indicates a possible role of smooth muscle cells and macrophages in local activation [Hansson *et al* 1989]. Furthermore, oxLDL induces the expression of HLA-DR and CD86 on human monocytes and promotes *in vitro* proliferation of T-lymphocytes [Fortun *et al* 2001].

1.9.1.3 Monocyte emigration in CAD

During resolution of inflammation, monocytes emigrate from the inflammatory site to peripheral lymph nodes and this process is impaired during atherosclerosis [Randolph 2008]. Emigration of monocyte derived cells is accompanied with lesion regression [Llodra *et al* 2004] and might involve interactions between ICAM-1 and CD11/CD18 [Randolph and Furie 1996].

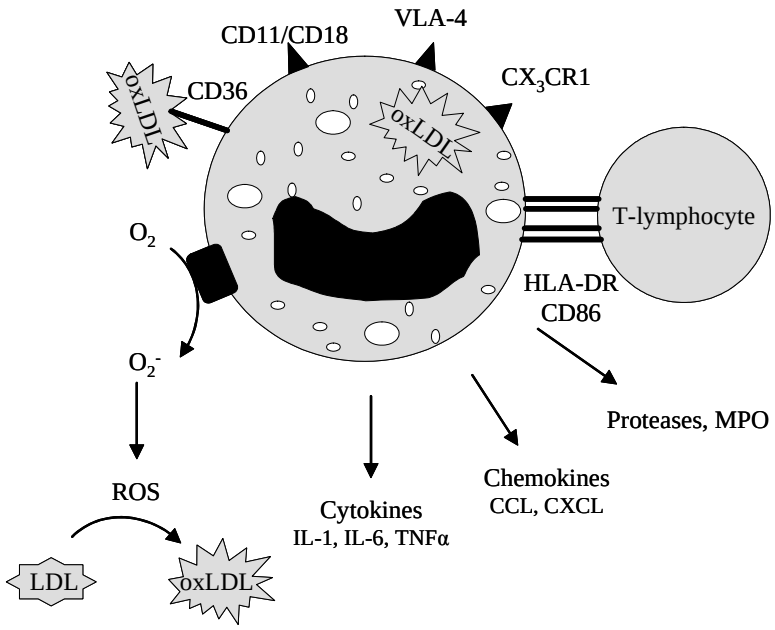

Figure 3. The role of monocytes in CAD with attention to markers studied in this thesis.

1.9.2 Neutrophils in CAD

The role of neutrophils in CAD has not been fully considered, however accumulating data indicates that neutrophils may contribute to the inflammatory reaction [Baetta and Corsini 2009]. A high number of circulating neutrophils is associated with an increased risk of CAD [Horne *et al* 2005, Haumer *et al* 2005] and the number of neutrophils correlates to the concentration of pro inflammatory molecules in circulation [Nijm *et al* 2005]. Neutrophils are detected at low numbers in fatty streaks in primates [Trillo 1982] and associate with acute coronary events in humans [Naruko *et al* 2002]. In a mouse model of atherosclerosis, neutrophils were the predominant leukocyte that interacted with atherosclerotic endothelium [Eriksson *et al* 2001]. Although increasing evidence suggests that neutrophils may infiltrate the atherosclerotic plaque, their short life span in tissue may limit their accumulation. A balance between CXCR2 and CXCR4 mediated signals has been suggested to regulate neutrophil extravasation to atherosclerotic lesions [Sainz and Sata 2008] since antagonists to CXCR4 promoted lesion formation in mice [Zernecke *et al* 2008]. Furthermore, antibody mediated depletion of neutrophils reduced the plaque area in mice [Zernecke *et al* 2008]. Together, these results imply that neutrophils contribute to the formation and progression of atherosclerotic plaques. Potential mechanisms for neutrophils in atherosclerosis may be by the release of cytokines, chemokines, MMPs and ROS.

1.9.2.1 Influence on leukocyte extravasation

Once activated, neutrophils secrete both CXCL and CCL chemokines and hence, induce the migration of many different leukocytes. In addition, granule proteins such as LL37 and heparin binding protein are chemotactic for monocytes [Soehnlein *et al*

2008]. Neutrophil interaction with endothelial cells can also induce opening of endothelial gap junctions, thereby promoting leukocyte extravasation. Hence, neutrophils can modulate the inflammatory milieu and the local recruitment of leukocytes to the plaque.

1.9.2.2 Generation of ROS in CAD

ROS, produced by endothelial cells and extravasated leukocytes, have profound effects on the initiation and progression of atherosclerosis. Neutrophils and monocytes that express MPO have been detected in the fibrous cap of unstable plaques [Tavora *et al* 2009]. Furthermore, the release of MPO during acute manifestations has been detected [Biasucci *et al* 1996] and the concentration of MPO in plasma correlates to the severity of CAD [Ndrepepa *et al* 2008]. ROS oxidize LDL and activate endothelial cells and smooth muscle cells. An increased production of ROS following PMA stimulation has been reported in patients with acute coronary disease [Takeshita *et al* 1997]. This is on the contrary to patients with stable CAD who have a lower production of ROS following PMA stimulation [Sarndahl *et al* 2007].

1.9.2.3 Release of matrix degrading proteases

The release of proteases from activated neutrophils constitutes one mechanism for breakdown and rupture of the fibrous cap. This is indicated by the association of neutrophils with ruptured plaques. The activity of MMP-9 is regulated by the formation of a complex with neutrophil gelatinase-associated lipocalin (NGAL) [Yan *et al* 2001]. Both NGAL and MMP-9 are expressed in acute CAD and associate with plaque rupture [Hemdahl *et al* 2005, Fukuda *et al* 2006].

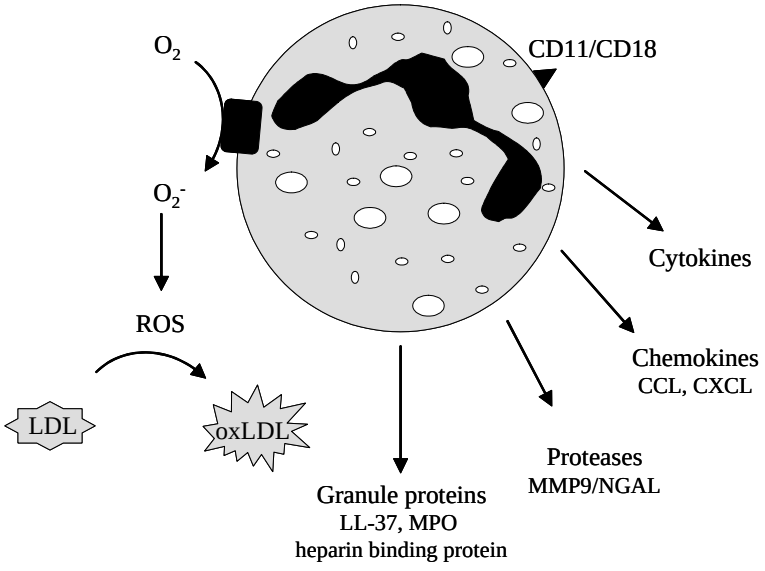

Figure 4. The role of neutrophils in CAD with attention to markers studied in this thesis.

1.9.3 Integrin profile in CAD

The expression of CD11b on circulating neutrophils and monocytes is increased during acute phases of CAD, which indicates cell activation [Meisel *et al* 1998, Lindmark *et al* 2001]. In addition, patients with acute MI have a higher expression of CD11a, VLA-4 and ICAM-1 on the circulating monocytes [Meisel *et al* 1998]. The expression and the affinity of CD11b are not altered during stable disease [Lindmark *et al* 2001, Sarndahl *et al* 2007]. Supporting data for a link between integrin expression and atherosclerosis is indicated by bone marrow transfer in CD18 knock out atherosclerotic mice. This experiment indicated that CD18 could be protective during fatty streak formation and pro-atherogenic in established mature lesions [Merched *et al* 2009].

2 AIMS

The objective was to study leukocyte *in vivo* extravasation in patients with CAD and healthy controls.

The specific aims were:
- to study if monocyte extravasation was altered in patients with CAD and hence could explain accumulation of monocyte derived cells in the plaque (**I**).

- to study if extravasated monocytes were functionally different in patients with CAD compared to healthy controls (**II**).

- to examine the hypothesis of neutrophil priming in patients with CAD by assessing the responsiveness and activation of peripheral and extravasated neutrophils (**III**).

- to study if extravasated neutrophils from patients with CAD could contribute to an enhanced pro-atherosclerotic inflammatory milieu (**IV**).

3 METHODS

This is a general overview of the methods used in the present thesis. For detailed descriptions please refer to each individual article.

3.1 PATIENT CHARACTERISTICS

Patients with stable CAD were recruited from the Karolinska University Hospital. All patients had a history of previous MI or unstable angina (UA), angiograpically confirmed atherosclerosis in as least two vessels and normal levels of creatinine in the serum.

	Females / Males	**Age (years)**	**MI / UA**	**Delay (months)**
I	12 / 7	61 (53-67)	17 / 2	8 (6-12)
II	7/11	62 (52-66)	17/1	11 (8-17)
III	9/4	59 (34-78)	12/1	12 (6-16)
IV	2/8	62 (58-64)	10	9 (8-10)

*Table 2. Characteristics of patients included in **paper I-IV**.*

The healthy controls were matched for gender and age ±3 or ±5 years. Patients and controls with known active inflammatory diseases (other than atherosclerosis), infections, diabetes mellitus or rheumatic diseases, as well as those receiving medical therapy with antibiotics, corticosteroids, immunosuppressive agents or Warfarin were excluded. All subjects gave informed consent and the studies were approved by the local ethical committee at the Karolinska University Hospital.

3.2 *IN VIVO* EXTRAVASATION, THE SKIN BLISTER METHOD

Skin blisters were induced on the forearm by a vacuum of 300 mmHg and heating at 39°C for 2-3 hours. The following morning (after approximately 14 hours), the blister exudates were aspirated and the blister areas were washed with PBS that was added to the collected samples (un-stimulated blister). The blister roofs were removed and sterilized open-bottom plastic skin chambers were mounted over the exposed blister floor. The chambers were filled with PBS (intermediately stimulated blister) or autologous serum (intensely stimulated blister), both containing heparin. After 9-10 hours, the chamber exudates were collected and the skin chambers were washed with an equal volume of PBS that was added to the collected sample. The chamber exudates were centrifuged and the supernatants were frozen and analyzed later. The cell pellets were dissolved in cell culturing medium and directly analyzed.

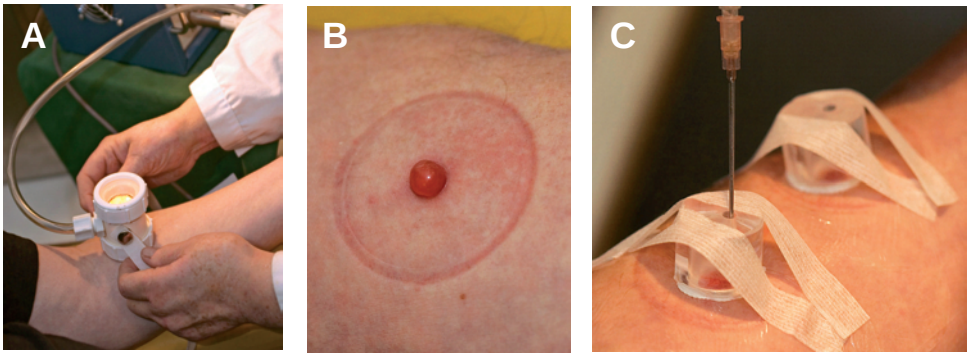

Figure 5. The skin blister method. Skin blisters are induced by vacuum and gentle heating (A). The un-stimulated blister exudate is collected the following morning after approximately 14 hours (B). After removal of the epidermal roof, skin chambers are mounted over the wound and filled with either PBS or autologous serum, representing the intermediately and intensely stimulated blisters (C).

3.3 ADDITIONAL METHODS

Binding of acLDL (**II**)	Performed by incubation with acLDL conjugated to Alexa Fluor 488 and measured by flow cytometry.
Electron microscopy (**IV**)	Enables visualization of cell morphology and antibody based staining of specific markers. Performed at the electron microscopy unit, Karolinska Institutet.
Ficoll Paque technique (**II, IV**)	Separation of neutrophils and mononuclear cells by density centrifugation.
Flow cytometry - Analysis of surface markers (**I-IV**)	Epics Elite (Beckman Coulter). Performed by labeling the leukocytes with fluorochrome conjugated antibodies.
- Evaluation of cell count (**I-IV**)	Determines total cell count and distribution of individual cell types.
Immunoassay (**I, III, IV**)	Determines the concentration of pro-inflammatory molecules in serum and chamber exudates by commercially available assays.
In vitro activation (**I-IV**)	Studied following stimulation with fMLP or PMA.

In vitro incubations - With serum and chamber exudate (**I, II**)	Performed on leukocytes from peripheral blood to evaluate the impact of the inflammatory milieu.
- With cytokines (**IV**)	Neutrophil activation by IL-1 was analyzed by incubations with recombinant human IL-1 after pre-treatment with IL-1R antagonist (IL-1Ra).
In vitro transmigration (**I**)	Assessed by the transwell method using inserts coated with extracellular matrix.
Microarray (**IV**)	Provides a thorough evaluation of gene expression. Performed at the microarray facility unit, Karolinska Biomics Center.
Oxidative burst (**III**)	Assessment of intracellular H_2O_2 production by the 2',7'-dichlorofluorescein diacetate (DCFH-DA) system and flow cytometry.
Purification of RNA (**IV**)	Extraction of RNA from purified leukocyte populations by the Qiagen RNeasy kit.
RT-PCR (**IV**)	Enables expressional analysis of individual genes. The Taq-Man system was used and genes were detected by means of the FAM-dye labeling system with commercially available primers. The results were normalized against a housekeeping gene and the relative expressions were calculated using the equation $2^{-\Delta\Delta Ct}$.
Statistical analyses (**I-IV**)	Comparisons between groups were made by use of Mann-Whitney U test. Correlations were made by Spearman's correlation analysis. A $P<0.05$ was considered significant.
Validation of microarray (**IV**)	Comparative analysis was carried out using GeneSpring™ (7.3). Data was normalized per chip and per gene. The expression profiles from three independent experiments were compared using ANOVA parametric test, assume variances equal.

4 RESULTS AND DISCUSSION

4.1 GENERAL CHARACTERIZATION OF THE SKIN BLISTERS (I-IV)

The numbers of extravasated leukocytes, collected with the skin chamber method, are presented in figure 6 which was published in **paper I**. Similar numbers were detected in **paper II-IV**.

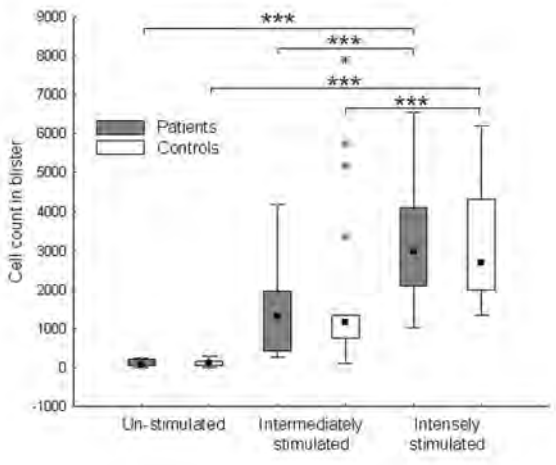

Figure 6. Numbers of transmigrated leukocytes ($x10^3$) in the un-stimulated, intermediately stimulated (PBS) and intensely stimulated (serum) blisters. No significant differences between patients and controls were detected. The numbers of transmigrated cells increased significantly with the degree of inflammation.
**** P<0.005 between different blister compartments.*

The median percentages of granulocytes, monocytes and lymphocytes in peripheral blood and in the exudates from healthy controls are presented in table 3. Data is adapted from **paper I** and represents a common distribution. No significant differences in the distribution of extravasated cells were detected between patients with CAD and healthy controls.

	Granulocytes %	**Monocytes %**	**Lymphocytes %**
Blood	60 (52-66)	11 (8.2-12)	30 (26-36)
Un-stimulated blister	59 (40-74)	34 (19-46)	7.7 (5.4-12)
Intermediately stimulated blister	81 (70-87)	13 (9.9-18)	3.5 (2.3-6.9)
Intensely stimulated blister	81 (77-86)	14 (10-20)	2.3 (1.3-6.6)

Table 3. The median percentage of granulocytes, monocytes and lymphocytes in peripheral blood and the skin blisters. Data represents a common distribution in healthy subjects.

The numbers and distributions of leukocytes in the skin chamber exudates are in the same range as in other publications [Forsgren and Scheja 1985, Hellum and Solberg 1977]. The number of extravasated leukocytes increased with the degree of inflammation and was highest in the intensely stimulated blister. The un-stimulated blister had a higher percentage of monocytes compared to the stimulated chamber exudates which is in agreement with a slower accumulation of monocytes. The un-stimulated blister is incubated overnight for approximately 14 hours while the skin chambers are incubated for 9-10 hours. All exudates were dominated by neutrophils and this is in agreement with previous publications. Figure 7 (from a manuscript not included in the thesis) views the morphology of neutrophils from blood and chamber exudates assessed by electron microscopy. The pictures indicate that extravasated neutrophils have an intact morphology with minor alterations in granule distribution.

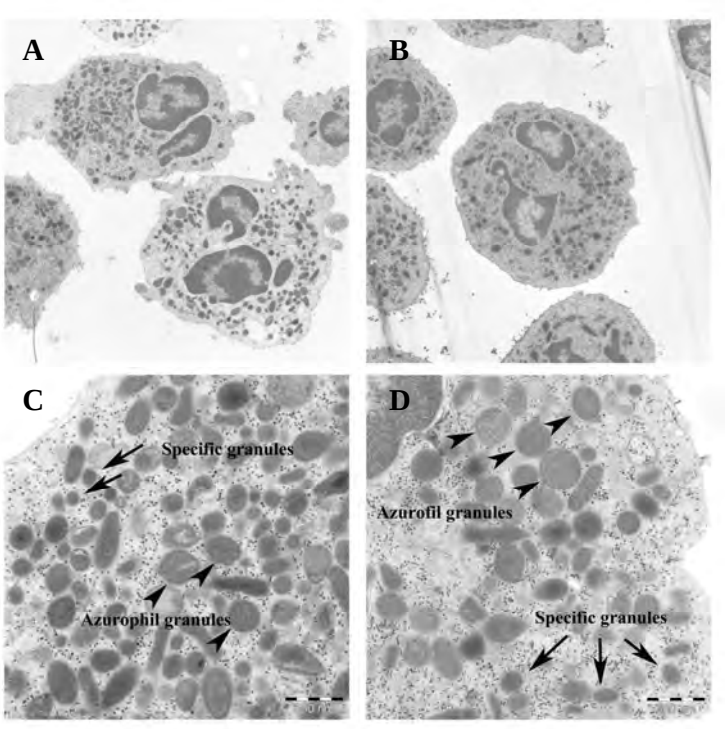

Figure 7. Electron microscopy of peripheral neutrophils (A, C) and extravasated neutrophils (B, D) from a healthy individual.

The concentrations of inflammatory markers in the exudates and in serum are presented in table 4, which is based on immunoassays from paper **I**, **III** and **IV**. Higher concentrations of inflammatory markers were detected in the chamber exudates compared to in serum and in addition increased with the intensity of inflammation. This confirms two separate inflammatory milieus. The numbers of extravasated leukocytes correlated to the ratios of CCL2 and CCL3 concentrations between blister exudates and serum (**paper I**) which indicates the importance of a gradient.

		Serum		Intermediate blister		Intense blister	
		CAD	Healthy	CAD	Healthy	CAD	Healthy
IL-6 (I)	(pg/ml)	1.5 (1.1-4.8)	1.3 (0.9-2.0)	5.2 (2.3-20) $\times 10^3$	8.8 (2.8-14) $\times 10^3$	9.7 (3.1-38) $\times 10^3$	12 (6.0-20) $\times 10^3$
IL-6 (III)	(pg/mL)	1.5 (1.1-5.2)	1.8 (1.2-3.5)	10 (3.6-22) $\times 10^3$	7.3 (2.8-9.1) $\times 10^3$	10.3 (7.5-20) $\times 10^3$	9.9 (5.6-14) $\times 10^3$
IL-8 (III)	(pg/mL)	6.8 (3.7-12)**	1.3 (1.3-5.4)	13 (6.0-25) $\times 10^3$	7.8 (3.5-14) $\times 10^3$	25 (17-35) $\times 10^3$	25 (15-33) $\times 10^3$
IL-10 (I)	(pg/ml)	0.7 (0.3-1.2)	0.5 (0.3-0.9)	37 (30-80)	38 (23-53)	45 (39-78)	66 (45-76)
TNF-α (I)	(pg/ml)	1.6 (1.3-3.6)	1.4 (0.7-1.9)	290 (160-1200)	190 (120-420)	430 (220-940)	450 (240-1000)
IL-1α (IV)	(pg/ml)	<1.0	<1.0	ND	ND	52 (37-83)	58 (43-120)
IL-1β (IV)	(pg/ml)	<1.0	<1.0	ND	ND	300 (210-1100)	200 (51-380)
IL-1Ra (IV)	(ng/ml)	0.3 (0.2-0.3)	0.2 (0.1-0.4)	ND	ND	18 (17-40)	12 (8.2-31)
IL-1R2 (IV)	(ng/ml)	12 (11-15)	11 (10-13)	ND	ND	17 (14-21)	16 (14-18)
CCL2 (I)	(ng/ml)	0.4 (0.3-0.5)	0.3 (0.2-0.4)	9.5 (4.8-15)	4.7 (2.6-12)	11 (6.1-18)	8.7 (5.5-17)
CCL3 (I)	(pg/ml)	32 (31-35)	30 (29-31)	2.8 (0.9-5.1) $\times 10^3$	1.1 (0.7-2.3) $\times 10^3$	1.4 (0.8-2.4) $\times 10^3$	1.6 (1.0-2.9) $\times 10^3$
CCL3 (IV)	(pg/ml)	20(18-22)	20 (18-20)	ND	ND	5.4 (1.8-6.8) $\times 10^3$	2.3 (0.9-3.9) $\times 10^3$
CCL4 (IV)	(pg/ml)	92 (61-180)	70 (65-87)	ND	ND	13 (8.6-21) $\times 10^3$	7.3 (3.9-16) $\times 10^3$
CCL20 (IV)	(pg/ml)	17 (8.7-37)	11 (7.4-14)	ND	ND	2.4 (2.0-4.2) $\times 10^3$	1.2 (0.7-4.0) $\times 10^3$
MMP9/NGAL (III)	(ng/ml)	31 (17-43)***	7.7 (4.4-15)	200 (79-270)*	55 (29-120)	280 (180-580)	280 (180-530)

*Table 4. The concentrations of inflammatory markers in exudates and serum. * P<0.05, ** P<0.01, *** P<0.005 between patients with CAD and healthy subjects.*

4.2 MONOCYTE EXTRAVASATION AND EXPRESSION OF CD11B (I)

Monocytes are the dominating cell type that extravasate into the atherosclerotic plaque. The main objective in this paper was to evaluate if patients with CAD had an altered extravasation of monocytes. The expression and mobilization of adhesion molecules and chemokines related to monocyte extravasation were studied.

Patients with CAD had similar numbers of monocytes as healthy controls in both blood and chamber exudates. This indicates that there is no enhanced extravasation of monocytes during stable CAD. This is in line with the expression of adhesion molecules on circulating monocytes and the ratio of chemokines that did not differ between patients and controls. The data is further supported by similar *in vitro* transmigration over laminin coated inserts. Median transmigration was 13 (10-19) % in patients and 8 (6-23) % in controls and did not differ between the groups. Altogether, this indicates that stable CAD patients have a comparable monocyte extravasation into local inflammatory sites as healthy subjects.

4.2.1 Expression of CD11b

CD11b is important during inflammation since it binds to ICAM-1 on activated endothelium and to extracellular matrix. In contrast to stable CAD, acute phases of CAD are associated with an increased expression of CD11b on circulating monocytes [Meisel *et al* 1998, Lindmark *et al* 2001]. In the present study, the expression of CD11b on circulating monocytes did not differ between the groups (figure 8), which confirms that the patients were in a stable state of CAD. Acute CAD is also associated with increased serum levels of inflammatory markers [Luo *et al* 2004, Mizia-Stec *et al* 2003]. The similar concentrations of CRP, IL-6 and TNF-α in the patients compared to controls further indicate a stable disease and are likely related to interventions with statins [Luo *et al* 2004].

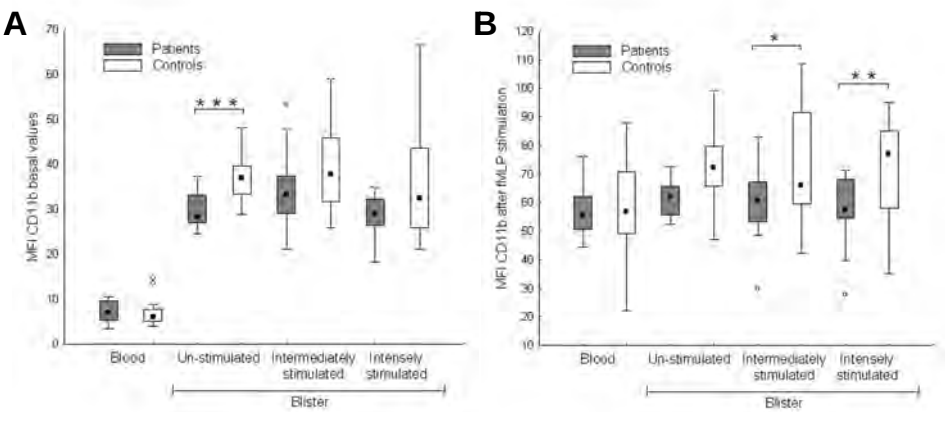

Figure 8. Basal expression of CD11b on peripheral and transmigrated cells (A). CD11b mobilization after stimulation with fMLP (B). P=0.002 in the un-stimulated blister between patients and controls (A). P=0.04 in the intermediately stimulated blister and P=0.006 the intensely stimulated blister following fMLP stimulation (B).

Despite similar expression of CD11b on circulating monocytes, patients with CAD had significantly lower expression of CD11b following extravasation. Figure 8 illustrates the basal expression of CD11b (figure 8A) and the expression of CD11b following fMLP stimulation (figure 8B). In order to discriminate the impact of extravasation, the expression of CD11b on extravasated monocytes was normalized to the expression on peripheral monocytes. The ratio of CD11b expression between extravasated monocytes and peripheral monocytes hence illustrates the mobilization of CD11b following extravasation. Table 5 illustrates the differences in CD11b ratio between patients and controls.

	Un-stimulated	**significance**	**fMLP-stimulated**	**significance**
Un-stimulated blister	CAD = Healthy	0.051	CAD = Healthy	NS
Intermediately stimulated blister	CAD < Healthy	0.04	CAD < Healthy	0.008
Intensely stimulated blister	CAD < Healthy	0.02	CAD < Healthy	0.003

Table 5. Differences between patients with CAD and healthy subjects regarding the ratio of CD11b expression between extravasated monocytes and peripheral monocytes. Non-significant (NS).

The expressions of CD11b following extravasation to the stimulated chambers were altered in patients compared to healthy subjects. This was seen both in un-stimulated and the fMLP stimulated cells. No differences were seen on monocytes from the un-stimulated blister. This blister is incubated for 14 hours while the intermediately and intensely stimulated chambers are incubated for 10 hours. In addition, the inflammatory milieu in the stimulated chambers differs from the un-stimulated blister. While the chambers are stimulated with PBS or serum, the un-stimulated blister is a reaction to blister formation alone. Differences in the local inflammatory milieu as well as the duration of incubation are likely to modulate the expression of CD11b.

CD11b is localized in the plasma membrane and in granules that are mobilized following stimulation [Miller *et al* 1987]. Although the concentrations of inflammatory mediators were similar between the groups (table 4), one can not rule out that other factors, such as C5a, could be altered in patients with CAD. Heterologous desensitization could affect the lower expression of CD11b following fMLP stimulation but does not explain the reduced expression on un-stimulated cells. It is important to consider the effect of statins that reduce the expression of CD11b in hypercholesterolemic patients [Serrano *et al* 2001]. It is possible that the results are affected by medical interventions, but if this was the only explanation, a more homogenous reduction of CD11b would be expected. In addition, the concentrations of cholesterol in the blood did not correlate to CD11b expressions. The altered CD11b expression in the patients was neither an effect of an altered viability, as indicated by staining with annexin V and propidium iodide.

Human alveolar macrophages do not mobilize CD11b following fMLP or PMA stimulation [Miller *et al* 1987]. A possibility is therefore that extravasated monocytes

from patients with CAD have a more differentiated phenotype which reduce the mobilization of CD11b. The $CD14^{+}CD16^{+}$ monocytes are considered to be more differentiated, and an increased number of these cells in peripheral circulation is associated with CAD [Schlitt *et al* 2004, Heine *et al* 2008]. Markers related to monocyte maturation were further studied in **paper II**.

In order to evaluate if the chamber fluid influenced the expression of CD11b, leukocytes from healthy subjects were incubated with skin chamber fluid for two hours. Chamber fluid from patients with CAD did not alter the expression of CD11b compared to chamber fluid from healthy controls. The time point might have been too short and it is possible that leukocytes from patients with CAD might have responded differently. However, no direct influence on CD11b expression was detected on monocytes from healthy subjects.

The altered profile of CD11b in CAD is likely an integrated outcome from several factors. In order to determine if interaction with extracellular matrix could influence CD11b expression, *in vitro* extravasation was performed. Monocytes were induced to migrate over laminin coated membranes towards CCL2, since CD11b/CD18 is involved in this migration [Jiang *et al* 1994]. The expression of CD11b on *in vitro* transmigrated monocytes was similar between patients and controls. Hence, interaction with laminin and *in vitro* migration towards CCL2 did not induce an altered expression of CD11b in patients with CAD compared to healthy subjects.

The concentration of proteases in the skin chamber exudate is not fully known but they may modulate the expression of CD11b. MMP-9/NGAL was increased in the intermediately stimulated chamber exudate in patients with CAD compared to healthy controls (table 5) which could indicate increased proteolysis in CAD. Proteolytic degradation of CD11b following a strong stimulation has been demonstrated [Davey *et al* 2000]. It is possible that cleavage of CD11b contributes to the lower expression of CD11b on extravasated neutrophils in patients with CAD.

Heparin is used as an anticoagulant in the skin chambers and this may influence the expression of CD11b since heparin is a ligand for CD11b/CD18 [Takada 2007]. The expression of CD11b was therefore analyzed in the presence of heparin, at a similar concentration as in the skin chamber. The results indicated no influence of heparin on CD11b expression neither in un-stimulated nor in fMLP stimulated cells.

Although the mechanism behind the observed dysregulation of CD11b in CAD is not known at present, it might have important biological consequences. During resolution of inflammation, monocytes emigrate from the inflammatory locus [Randolph 2008], and this characterizes plaque regression [Llodra *et al* 2004]. The process of reverse migration is mediated by ICAM-1 and β_2 integrins [Randolph and Furie 1996]. A lower expression of CD11b following extravasation might therefore contribute to monocyte entrapment at local inflammatory sites.

4.2.2 Expression of VLA-4

VLA-4 is also important for monocyte extravasation during inflammatory conditions. The expression of VLA-4 on peripheral monocytes is slightly increased during acute CAD [Meisel *et al* 1998]. Our results indicated no significant difference in VLA-4 between patients and controls on either peripheral or extravasated monocytes. The expression of VLA-4 was decreased following extravasation, which might be an effect of shedding. Shedding of LFA-1 in a blister model concomitant with a non-significant reduction in VLA-4 has been reported [Evans *et al* 2006].

4.3 THE PHENOTYPE OF EXTRAVASATED MONOCYTES (II)

The main objective in this paper was to evaluate if extravasated monocytes were functionally different in patients with CAD compared to healthy controls. Markers associated with the role of monocytes/macrophages in CAD were analyzed. Co-expression of CD14 and CD16 was studied since the $CD14^+CD16^+$ monocytes are associated with CAD. Further assessments included expression of CX_3CR1 that mediates extravasation of $CD14^+CD16^+$ monocytes, CD86 and HLA-DR that regulate antigen presentation, CD36 that is deeply involved in foam cell formation, and the binding of acLDL.

4.3.1 Accumulation of $CD14^+CD16^+$ monocytes

The number of circulating $CD14^+CD16^+$ monocytes correlates to the thickness of the carotid intima media [Rogacev *et al* 2009] and the prevalence of CAD [Schlitt *et al* 2004, Heine *et al* 2008]. In our study, patients with CAD had similar numbers of $CD14^+CD16^+$ monocyte compared to healthy controls in both blood and chamber exudate. This could reflect that the patients were in a stable phase and under the influence of medical interventions.

The median percentage of $CD14^+CD16^+$ monocytes in the chamber exudates was 40% compared to 10% in peripheral blood. This could indicate either accumulation of $CD16^+$ monocytes or induction of CD16 by local factors in the sin chamber and by adhesive interactions during extravasation. The expression of CD16 can be induced by *in vitro* incubation with M-CSF or GM-CSF in presence of IL-4 and IL-10 [Li *et al* 2004, Allavena *et al* 1998]. In addition, P-selectin has been shown to induce maturation of CD16 positive dendritic-like cells [Li *et al* 2003]. Maturation of CD16 positive cells *in vitro* is vastly influenced by the specific settings and does not truly reflect formation of the $CD16^+$ subtype *in vivo*. $CD14^+CD16^+$ monocytes from peripheral blood have a genotype and a phenotype that partly resembles those in both macrophages and dendritic cells [Ancuta *et al* 2009]. Hence, the $CD14^+CD16^+$ subtype is considered to be more mature.

Significantly increased concentrations of IL-10 (table 4) and GM-CSF [Kuhns *et al* 1992] have previously been detected in chamber exudate. We therefore tested whether the chamber fluid could induce the expression of CD16 on peripheral monocytes. CD16 was not induced following 18 hours of incubation, favoring the idea that adhesion-mediated events might be crucial in the induction of CD16. Alternatively,

CD16 positive monocytes are selectively recruited to the skin chamber. Furthermore, it can not be ruled out that the $CD14^{+}CD16^{+}$ monocytes have an altered emigration from the skin chamber, favoring accumulation of this subtype.

4.3.2 Expression of CX_3CR1

The recruitment of $CD14^{+}CD16^{+}$ monocytes to inflammatory sites is particularly associated with fractalkine. Figure 9 views the expression of CX_3CR1, CD86, HLA-DR and CD36 on monocytes from peripheral blood and skin chamber exudate.

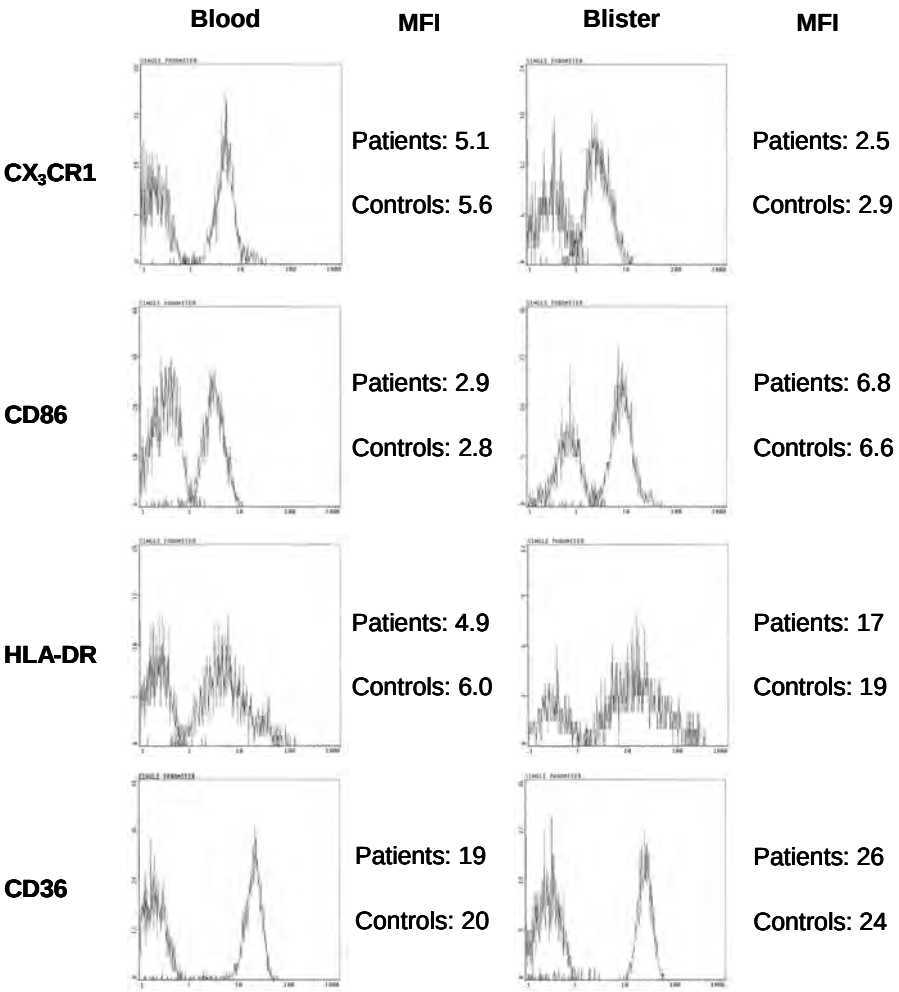

Figure 9. The expressions of CX_3CR1, CD86, HLA-DR and CD36 assessed by flow cytometry. The cytograms view the binding of the isotype and the specific antibody. The median MFI for patients and controls are indicated to the right of each cytogram. No significant differences were detected between patients and controls.

More than 95% of the circulating monocytes expressed the fractalkine receptor CX_3CR1. In agreement with a previous publication, the expression of CX_3CR1 was similar between CAD patients and healthy subjects [Damas *et al* 2005]. Following extravasation, the expression of CX_3CR1 was significantly reduced, which may indicate cleavage during extravasation. A lower expression of CX_3CR1 on lymphocytes from synovial fluid compared to peripheral blood has previously been demonstrated [Ruth *et al* 2001] and may indicate a general down- regulation of CX_3CR1 following extravasation.

4.3.3 Markers associated with antigen presentation

The expression of HLA-DR and the co-stimulatory molecule CD86 increased following extravasation (Figure 9). HLA-DR is associated with maturation of mononuclear cells into antigen-presenting cells. In order to determine if the skin chamber exudate could influence the expression of HLA-DR, *in vitro* incubations were performed. Incubation of peripheral monocytes with chamber exudate did not induce HLA-DR. $CD16^+$ monocytes have an increased presentation of antigens that coincide with increased expressions of HLA-DR and CD86 [Skrzeczynska-Moncznik *et al* 2008]. The higher expression of HLA-DR on extravasated monocytes may therefore be closely linked to accumulation of $CD16^+$ monocytes. However, this can only be speculated upon, since multicolor analysis of CD14, CD16 and HLA-DR was not performed.

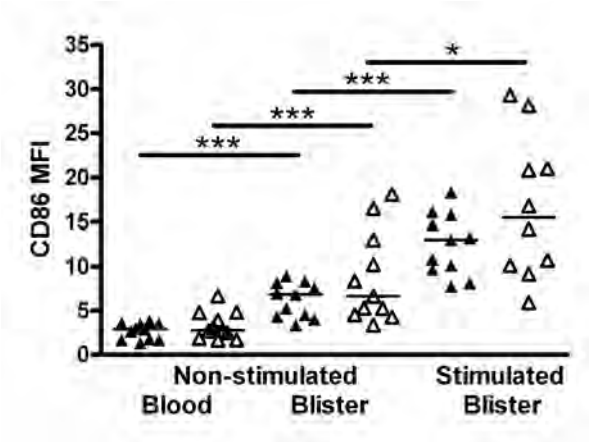

Figure 10. The Expression of CD86 on monocytes from peripheral blood and chamber exudate. The expression was increased following extravasation and could be further induced by stimulation with fMLP. No significant differences were detected between patients and controls. Filled triangles refer to CAD patients and open triangles refer to healthy controls.

The expression of CD86 was higher on extravasated monocytes compared to circulating monocytes and could be further induced by *in vitro* stimulation with fMLP, indicating a pool of mobilized CD86 (Figure 10). The increased expression of CD86 following *in vitro* fMLP stimulation may also suggest a linkage between fMLP mediated innate and adaptive immune responses. $CD16^+$ monocytes have an increased

expression of CD86 [Skrzeczynska-Moncznik *et al* 2008] which may contribute to the higher expression of CD86 on exudated monocytes.

An increased expression of CD86 has been detected on monocyte derived dendritic cells from patients with CAD compared to healthy controls [Dopheide *et al* 2007]. However, these cells were cultivated *in vitro* for nine days. Our results suggest that there is no direct alteration in CD86 expression following extravasation in patients with CAD compared to healthy controls. It is however possible that changes between patients with CAD and healthy controls may commence following differentiation and maturation of antigen-presenting cells.

4.3.4 Expression of CD36 and binding of acLDL

CD36 is the main receptor for oxLDL and highly involved in foam cell formation [Endemann *et al* 1993]. The expression of CD36 is induced during differentiation [Huh *et al* 1996] and following adhesion to TNF-activated endothelial cells [Huh *et al* 1995]. A modest increase in the expression of CD36 was detected on extravasated monocytes compared to circulating monocytes (figures 9 and 11) which may account for interactions during extravasation as well as maturation. There was no difference between patients and controls. The expression of CD36 is induced by both naïve and modified LDL [Han *et al* 1997]. It can only be speculated on if the reduced concentration of plasma LDL in the patients influenced the expression of CD36. In addition, one can not rule out cleavage of CD36, since an elevated concentration of soluble CD36 is detected in patients with type 2 diabetes [Handberg *et al* 2006].

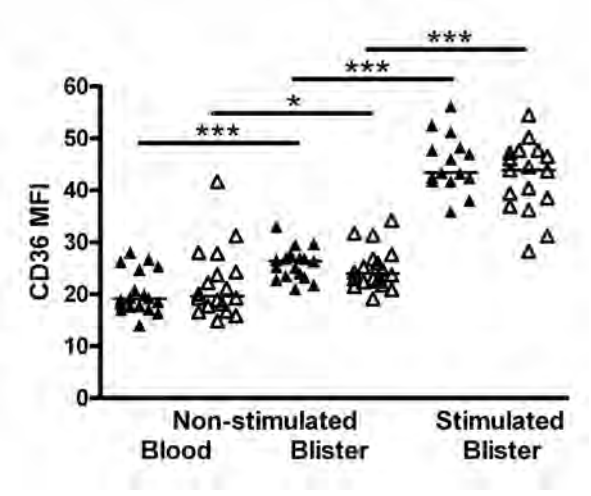

Figure 11. The expression of CD36 on monocytes from peripheral blood and chamber exudate. The expression was increased following extravasation and could be further induced by stimulation with fMLP. No significant differences were detected between patients and controls. Filled triangles refer to CAD patients and open triangles refer to healthy controls.

The expression of CD36 on extravasated monocytes was further induced by *in vitro* stimulation with fMLP (Fig 11). CD36 binds to thrombospondin-1 and increased thrombospondin binding that coincides with degranulation is detected on stimulated human neutrophils [Suchard *et al* 1992]. Together, this might indicate an intracellular pool of CD36 that can be mobilized.

The expression of CD36 following fMLP stimulation might be influenced by monocyte/platelet aggregates. Mononuclear cells were therefore separated over ficoll and then subjected to repeated low g-force washes prior to fMLP stimulation. This method has previously been shown to markedly reduce the number of platelets. The results indicated that platelets had no influence on the expression of CD36.

The expression of CD36 was further assessed following *in vitro* incubation with chamber fluid. The results were normalized against the expression of CD36 on monocytes that were incubated with cell medium alone. Blister fluid from healthy subjects did not alter the expression of CD36, the ratio to un-stimulated cells was 0.98 (0.79-1.16). Chamber fluid from patients with CAD significantly induced the expression of CD36. The median ratio between chamber fluid from CAD patients and cell medium was 1.25 (1.16-1.36). This indicates that the inflammatory milieu in patients with CAD might influence the expression of CD36. Factors in the skin chamber fluid that could induce CD36 include M-CSF and IL-4 [Yesner *et al* 1996].

The binding of modified LDL was assessed by flow cytometry on cells that had been incubated with acLDL conjugated to a fluorescent dye. The binding of acLDL was significantly higher on extravasated cells (20 (17-24) MFI) compared to cells from peripheral circulation (8.8 (7.8-10) MFI) in healthy subjects. No significant differences were detected between patients and controls. Binding of acLDL can be mediated by CD36 as well as SR-A1 [Kunjathoor *et al* 2002]. The expression of SR-A1 was assessed by flow cytometry. The expression of SR-A1 was comparable in blood (29.4 (23.9-35.1) %) and chamber exudate (29.3 (17.45-39.45) %) in healthy controls and did not differ to patients. The higher proportion of $CD16^+$ monocytes in the exudate compared to in blood might affect the results on SR-A1 since $CD16^+$ monocytes have a lower expression of SR-A1 [Draude *et al* 1999]. However, the increased expression of CD36 following extravasation might associate with the enhanced binding of modified LDL.

4.4 THE FUNCTION OF EXTRAVASATED NEUTROPHILS (III)

The objective was to study activation of circulating and extravasated neutrophils. Soluble markers associated with neutrophil activation, IL-8 and MMP9/NGAL, were measured in serum and chamber exudates. Expression of CD11b and production of ROS were assessed as markers of functional-related activation.

A high number of neutrophils is associated with an increased risk and poor outcome of CAD [Horne *et al* 2005, Haumer *et al* 2005]. Furthermore, the number of neutrophils correlates to the concentration of pro-inflammatory molecules in circulation [Nijm *et al*

2005]. In the present study, similar numbers of neutrophils and concentrations of pro-inflammatory markers IL-6 and CRP were detected in patients with CAD compared to healthy controls. This may indicate that the patients were in a stable disease and in addition treated with statins that are endowed with anti-inflammatory effects [Luo *et al* 2004].

4.4.1 Concentration of IL-8 and MMP-9/NGAL

Markers associated with neutrophil activation, IL-8 and MMP9/NGAL, were significantly higher in serum from patients compared to healthy controls (table 4). This could indicate that circulating neutrophils are primed. An increased concentration of IL-8 in plasma has previously been reported in both stable and unstable CAD [Hashmi and Zeng 2006]. Furthermore, IL-8 is an independent predictor of disease outcome [Inoue *et al* 2008]. The increased concentrations of IL-8 and MMP-9/NGAL in CAD might be an integrated outcome of several factors. IL-8 is induced in vascular smooth muscle cells and endothelial cells following activation with IL-1β [Wang *et al* 1991]. In addition, shear stress induces the production of IL-8 and MMP-9 in endothelial cells [Cheng *et al* 2008, Sun *et al* 2007].

The expression of MMP-9/NGAL could be influenced by the release of gelatinase and specific granules [Kjeldsen *et al* 1994 a, and b]. NGAL is associated with neutrophil specific granules [Kjeldsen *et al* 1994 a] and extravasation to the skin chamber has previously been shown to mobilize 20% of the specific granules [Sengelov *et al* 1995]. However, the concentration of MMP-9/NGAL is not necessary linked to neutrophil degranulation or the concentration of individual components since most NGAL form monomers or dimmers that are not associated with MMP-9 [Kjeldsen *et al* 1993]. NGAL protects MMP-9 from degradation and an increased concentration of the complex could contribute to an increased activity of MMP-9 in CAD [Yan *et al* 2001]. A positive feedback between IL-8 induced release of MMP-9 and MMP-9 induced truncation of IL-8 exists, and truncated IL-8, compared to non-truncated, has an increased biological activity [Van den Steen *et al* 2000]. MMP-9 has been attributed to weakening of the fibrous cap and is a predictor of CAD outcome [Blankenberg *et al* 2003]. The concentration of MMP-9 is increased in patients with stable as well as acute CAD [Zeng *et al* 2005]

4.4.2 Expression of CD11b and production of ROS

The expression of CD11b and production of ROS were assessed to study neutrophil function (Table 6). Basal expression of CD11b and production of H_2O_2 were similar between patients and controls in both blood and exudated neutrophils. Following *in vitro* stimulation, extravasated neutrophils from the intensely stimulated chamber in patients with CAD had a significantly lower expression of CD11b and a lower production of H_2O_2 compared to healthy controls (table 6). A general trend towards a lower response following extravasation and concomitant *in vitro* stimulation was seen in the patients but was only significant in exudate from the intensely stimulated chamber.

	Un-stimulated		*In vitro* stimulated	
	CAD	**Healthy**	**CAD**	**Healthy**
		Expression of CD11b (MFI)		
Blood	4.3 (3.4-6.8)	4.7 (3.6-5.4)	81 (72-99)	85(75-110)
Intermediately stimulated chamber	53 (47-59)	62 (50-79)	113 (105-132)	138 (110-172)
Intensely stimulated chamber	53 (43-56)	67 (53-75)	99 (79-120)**	118 (105-143)
		Production of H_2O_2 (MFI)		
Blood	4.4 (2.9-8.1)	3.1 (1.9-6.3)	143 (106-189)	188 (165-222)
Intermediately stimulated chamber	6.6 (5.7-9.3)	7.0 (5.5-10.7)	90 (57-116)	139 (89-157)
Intensely stimulated chamber	4.6 (2.0-9.7)	7.5 (5.0-8.7)	94 (38-123)*	128 (95-199)

Table 6. The expression of CD11b and production of ROS in neutrophils from peripheral blood and chamber exudate. fMLP was used as an stimuli for assessment of CD11b expression and PMA was used as a stimuli for assessment of ROS production.
** P<0.05 and ** P<0.01 between patients and controls.*

Total and high affinity CD11b expressions on neutrophils from peripheral blood is similar in patients with stable CAD compared to healthy controls [Lindmark *et al* 2001, Sarndahl *et al* 2007]. This is in agreement with our results on un-stimulated peripheral and exudated neutrophils.

An increased production of ROS in circulating neutrophils from patients with acute CAD has been reported following PMA stimulation [Takeshita *et al* 1997]. On the contrary, a reduced production of ROS has been reported during stable CAD following stimulation with C3bi opsonised yeast [Sarndahl *et al* 2007]. In our study, there was a trend towards a lower production of ROS in circulating neutrophils from patients with CAD compared to healthy controls, however this was not significant. Possible discrepancies may be explained by differences in stimuli, duration and choice of measurement.

The main reservoir of fMLP receptors is localized in the easily mobilized secretory vesicles [Sengelov *et al* 1994] that also contain CR1. In order to test if the differences were induced by a reduced mobilization of secretory vesicles, CR1 was analyzed. The expression of CR1 on circulating neutrophils was 32 (24-46) % in patients and 27 (13-54) % in controls. Corresponding numbers on extravasated neutrophils was 99 (95-100) % and 99 (97-100) %. Thus, the expression of CD11b in patients with CAD is not explained by an altered mobilization of fMLP receptors. CD11b is mainly localized to specific and gelatinase granules [Sengelov *et al* 1993]. The specific granules are also a reservoir of the membrane bound part of the NADPH [Borregaard *et al* 1983]. The expression of CD11b on un-stimulated and stimulated neutrophils from blood indicates

similar mobilization of specific and gelatinase granules between patients and controls. Therefore, the observed differences in CD11b and H_2O_2 between patients and controls are likely not an effect of altered granule mobilization.

A potential priming of circulating neutrophils favors the hypothesis that patients with CAD should have an increased expression of CD11b and an increased production of H_2O_2 following activation. However, neutrophils can be primed in several ways: in circulation by an increased concentration of IL-8, during extravasation due to integrin mediated activation and in the chamber exudate by inflammatory mediators. IL-8 priming of fMLP induced oxidative burst is observed *in vitro* [Guichard *et al* 2005]. However, the mechanism of chronic exposure to IL-8 is not known and could also result in desensitization.

Following excessive stimulations with repeated stimuli that utilize similar intracellular pathways, neutrophils become desensitized. An important inflammatory mediator in the skin chamber is C5a [Follin *et al* 1991]. Heterologous desensitization between IL-8, C5a and fMLP has been reported [Richardson *et al* 1995, Tomhave *et al* 1994]. In addition, desensitization between IL-8 and the β_1-integrin ligand fibronectin has been reported [Stanton *et al* 1999]. Desensitization may hence occur at many different levels. PMA is a receptor independent stimulus that directly activates PKC. The fMLP receptor is however not desensitized by PKC mediated phosphorylation [Richardson *et al* 1995]. The observed response could be mediated by desensitization below PKC at the level of PLC or further downstream.

The expression of CD11b on circulating neutrophils from patients with stable CAD has been studied following stimulation with IL-8 or LTB_4 [Sarndahl *et al* 2007]. The former study did not indicate an altered response in patients with CAD. However, IL-8 and LTB_4 are less potent than fMLP. A hierarchy between different attractants exists with fMLP being an end target [Campbell *et al* 1997, Heit *et al* 2002]. The lower expression of CD11b in our study was only significant in extravasated neutrophils that were further stimulated *in vitro*. Desensitization is expected to be most pronounced following strong stimulation, such as stimulation of extravasated cells.

An additional mechanism for the reduced expression of CD11b in patients with CAD could be proteolytic shedding, which has previously been demonstrated following intense stimulation [Davey *et al* 2000]. Leukocyte activation is influenced by the composition of lipid rafts in the cellular membrane. One possible explanation is that statin-treated patients have an altered signaling due to changes in the lipid rafts. The importance of lipid rafts for activation and exocytosis has been reported in natural killer cells [Inoue *et al* 2002]. In addition, neutrophils from patients with myelodysplasia (a hematological disorder) have a reduced production of ROS that coincides with an impaired formation of lipid rafts [Fuhler *et al* 2007].

4.5 GENE EXPRESSION IN EXTRAVASATED NEUTROPHILS (IV)

The objective in this paper was to study if extravasated neutrophils could have an immuno-modulatory role at a local inflammatory site. Gene expression in extravasated neutrophils and the potential impact of IL-1 activation were assessed.

The number of circulating neutrophils was significantly increased in patients with CAD 3.8 (3.0-3.9) $x10^9$ neutrophils/μL compared to in healthy controls 2.6 (1.8-3.0) $x10^9$ neutrophils/μL. This finding is in line with previous studies that have shown a correlation between the number of circulating neutrophils and risk of CAD [Horne *et al* 2005, Haumer *et al* 2005].

Neutrophils are considered to be fully mature when they leave the bone marrow and are hence transcriptionally silent. However, induction of gene transcription occurs following neutrophil extravasation. Gene expression analysis of neutrophils from a chamber exudate has previously shown an induction of IL-1, NFκB, CCL3 and CXCL2, among others [Theilgaard-Monch *et al* 2004]. In addition, extravasated neutrophils from an endotoxin-induced inflammation in the lung have an induced expression of IL-1, IL-8, CCL2, CCL3 and CCL4, among others [Coldren *et al* 2006]. Corresponding genes are also induced following exposure to lipopolysaccharide [Malcolm *et al* 2003].

The expression of genes in purified neutrophils from peripheral blood and chamber exudates was studied with microarray, assessing $20x10^3$ genes. Pathway analysis indicated a general induction in the IL-1 axis following extravasation. Induction of IL-1R1, IL-1RN, NFκB, CCL3, CCL4, CCL20 and CXCL2 was further confirmed by RT-PCR (Figure 12A). These findings are in line with previous publications on extravasated neutrophils [Theilgaard-Monch *et al* 2004, Coldren *et al* 2006].

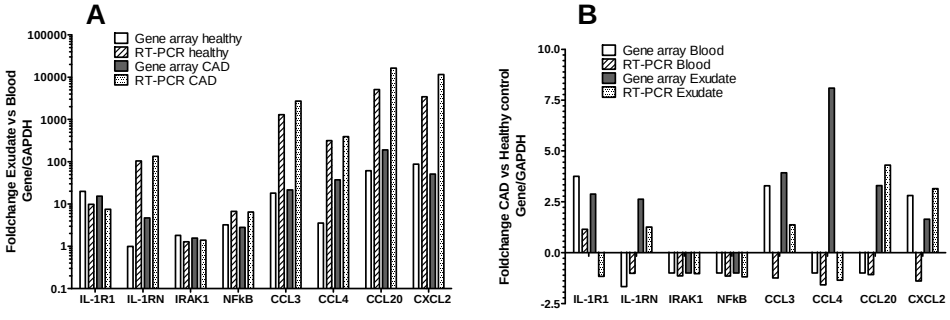

Figure12. Gene expression analyzed by gene array (n=3+3) and RT-PCR (n=7+7). Changes in gene expression between exudated and peripheral neutrophils (A). Changes between patients with CAD and healthy controls (B). Interleukin-1 receptor-associated kinase 1 (IRAK1).

IL-1 is essential for activation of endothelial cells and production of chemokines at local inflammatory sites [Pober and Sessa 2007] and an induced migration of

neutrophils has been demonstrated following IL-1 activation of endothelial cells [Furie and McHugh 1989]. Local IL-1 is also an important factor for leukocyte activation, IL-1 primes neutrophils for IL-8 induced responses [Brandolini *et al* 1996] and mediates a weak activation of NFκB in circulating neutrophils [McDonald *et al* 1997]. Stronger stimuli such as TNF-α and LPS have a greater impact on NFκB activation [McDonald *et al* 1997] and mediate induction of chemokines and cytokines [Cloutier *et al* 2007].

In vitro incubation of purified neutrophils from the blood of healthy subjects confirmed the induction of CCL3, CCL4, CCL20, CXCL2 and NFκB genes by IL-1. Expressions of CCL3 and CCL4 were further assessed by immunoassay on the supernatant from IL-1 stimulated neutrophils. IL-1 induced a low production and release of both chemokines. Our data indicate that IL-1 may activate neutrophil gene transcription and that IL-1 stimulated neutrophils constitute a source of chemokines.

In order to determine a potential role for the IL-1 axis in extravasated neutrophils, the expression of IL-1R1 was measured by flow cytometry and immuno electron microscopy. Figure 13 views the expression of IL-1R1 on neutrophils from peripheral blood and the chamber exudate.

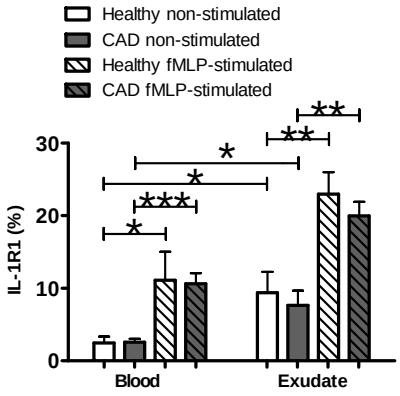

Figure 13. Expression of IL-1R1 assessed by flow cytometry. The expression of IL-1R1 was significantly increased following extravasation and could be induced by fMLP stimulation in both healthy controls and patients with CAD.

The expression of IL-1R1 on circulating neutrophils was low and could be significantly induced by fMLP. Extravasated neutrophils had an increased expression of IL-1R1 compared to circulating neutrophils and the expression could be further induced by fMLP. Immuno electron microscopy indicated an intracellular pool of IL-1R1. The expression of IL-1R on human neutrophils from peripheral blood has been estimated to 900 receptors per cell and internalization of radiolabeled IL-1 indicate that the receptors are functionally active [Parker *et al* 1989]. It is intriguing to speculate if the increased expression of IL-1R1 on extravasated neutrophils represents newly synthesized receptors. An increased gene expression of IL-1R1 was detected following extravasation. In addition, a higher number of IL-1R1 was detected in extravasated neutrophils compared to in circulating neutrophils assessed by immuno electron

microscopy. The intracellular location and induction of IL-1R1 following fMLP stimulation might indicate a pool of IL-1R1 that can be mobilized. However, soluble IL-1R1 has been demonstrated in serum and at an inflammatory location [Okamoto *et al* 2009, Arend *et al* 1994]. Whether the intracellular pool of IL-1R1 represents soluble receptors that have been phagocytosed is not known at present. The expression of soluble IL-1R1 in the chamber exudate was not measured. However, the concentration of soluble IL-1RII was slightly increased in the chamber exudate compared to in serum in both patients and healthy controls (table 4). Whether IL-1R1 follows the same expression profile as IL-1RII in the blister fluid can only be speculated on. Thus, the induced expression of IL-1R1 following fMLP stimulation might indicate the existence of mobilized IL-1R1.

The concentrations of IL-1α, IL-1β and IL-1Ra were increased in the chamber exudate compared to in serum in both patients and controls (table 4). Taken together, these results indicate that the IL-1 axis is activated following extravasation in both CAD patients and healthy subjects. Increased gene and protein expressions of IL-1β, IL-1R1 and IL-1Ra have been detected in specimens from atherosclerotic arteries [Olofsson *et al* 2009]. In addition, polymorphism in IL-1Ra is associated with CAD [Olofsson *et al* 2009]. Circulating mononuclear cells from patients with stable and acute CAD have increased expressions of IL-1α, IL-1β and IL-1Ra that are modulated by statins [Weahre *et al* 2004]. Similar concentrations of IL-1α, IL-1β, IL-1Ra and soluble IL-1RII were detected between patients and controls (table 4) and might be the result of medical interventions.

Minor differences in gene expression were noted in peripheral blood from patients with CAD, compared to healthy controls, 86 genes had an increased expression and 131 genes had a lower expression. Following extravasation, there was a higher expression of 400 genes and a lower expression of 1525 genes in patients compared to healthy controls. Pathway analysis indicated differences in the IL-1 axis in patients with CAD compared to healthy controls (Figure 14). The increased expression of CCL20 and CXCL2 in extravasated neutrophils from patients with CAD compared to healthy controls was confirmed by RT-PCR (Figure 12B).

An increased expression of CCL20 has been demonstrated in extravasated neutrophils from synovial fluid of patents with rheumatoid arthritis [Schlenk *et al* 2005]. CCL20 binds to CCR6 that is mainly expressed on dendritic cells and activated T- and B-lymphocytes [Baba *et al* 1997]. An increased production of CCL20 in CAD could be important for recruitment of these cells. The expression of CD1 [Melian *et al* 1999] and co-stimulatory molecules [de Boer *et al* 1997] in human atherosclerotic plaques indicate that antigen-specific T-lymphocytes might be involved in the inflammatory reaction.

Chemokines in the CXCL family are generally considered to activate neutrophils. However, CXCL2 mediates P-selectin dependent arrest of monocytes under flow [Smith *et al* 2005]. Furthermore, CXCR2, the receptor of CXCL2, is induced on monocytes by oxLDL [Lei *et al* 2002] and expression of CXCR2 is detected in human atherosclerotic lesions [Boisvert *et al* 1998]. Furthermore, studies in knock-out mice indicate that CXCR2 is important for macrophage accumulation and plaque progression

[Boisvert *et al* 1998]. An increased expression of CXCL2 at local inflammatory sites may therefore have an important function in accumulation of both neutrophils and monocytes.

The concentrations of CCL3, CCL4 and CCL20 were significantly higher in the exudate compared to in serum in both patients and controls (table 4). No significant differences were detected between patient and controls. The inflammatory milieu in the skin chambers is determined by the combined production of cytokines and chemokines from infiltrated leukocytes, endothelial cells and fibroblasts. Altered production from individual cell types might therefore be masked. In addition, statins reduce the expression and spontaneous release of chemokines [Waehre *et al* 2003, Veillard *et al* 2006]. This may be reasons for discrepancies between gene and protein expressions.

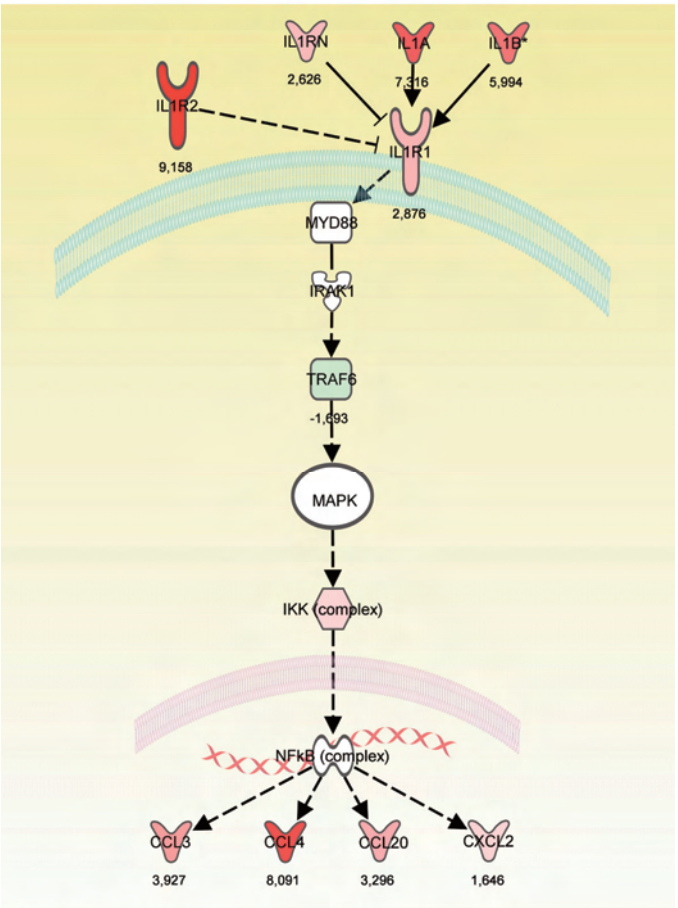

Figure 14. Changes in gene expression (>1.5x) in extravasated neutrophils from patients with CAD compared to healthy controls. Data was analyzed by the ingenuity software according to biological function. Red indicates an increased and green a lower expression in patients with CAD compared to healthy controls.

5 CONCLUSIONS

I. Patients with CAD had similar numbers of extravasated monocytes at the local inflammatory sites as healthy controls. However, compared to healthy subjects, extravasated monocytes from patients with CAD had a lower expression of the adhesion molecule CD11b. This may contribute to entrapment of monocytes at local inflammatory sites.

II. A higher percentage of $CD14^{+}CD16^{+}$ monocytes was observed in the skin chamber compared to in blood which might indicate accumulation of more mature cells. In addition, extravasated monocytes had increased expressions of markers associated with antigen presentation and foam cell formation. No differences were detected between patients and controls. However, the skin chamber fluid from patients with CAD induced the expression of CD36, favoring the concept of a pro-atherosclerotic local milieu in patients with CAD.

III. An increased expression of IL-8 was detected in serum from patients with CAD and might associate with peripheral priming of the neutrophils. The concentration of MMP-9/NGAL was increased in the circulation and at the local inflammatory site and this could contribute to the pathology in CAD. The responsiveness of extravasated neutrophils from patients with CAD was reduced compared to healthy controls. This might indicate desensitization and an altered intracellular activation in extravasated neutrophils in patients with CAD.

IV. Extravasated neutrophils had an increased expression of chemokines, and pathway analysis indicated activation of the IL-1 axis. The expression of IL-1R1 increased following extravasation, and *in vitro* stimulation with IL-1 resulted in expression of chemokines. Patients with CAD had increased expressions of CCL20 and CXCL2 following extravasation which could contribute to accumulation of mononuclear cells at a local inflammatory site. Hence, extravasated neutrophils may enhance the pro-inflammatory milieu.

6 FUTURE PERSPECTIVES

Several hypotheses have been highlighted in this thesis and with them, many more questions have followed. I would like to emphasize a few of those and the future perspectives that have arisen during this time.

The transmigration process is highly regulated at several different levels. Adhesion molecules are regulated by the avidity and the affinity and chemokines are regulated by the formation of gradients, truncations and by dimerisation. Although the high affinity conformation of CD11b did not differ on circulating neutrophils during CAD [Sarndahl *et al* 2007], it is appealing to investigate the expression following extravasation. The affinity of CD11b is regulated during transmigration by inside out signals and one approach is to measure the active epitope of CD11b in relation to total CD11b expression following extravasation. This could give additional information on adhesion molecule regulation in CAD.

In addition, the increased concentration of proteases in the local inflammatory milieu in patients with CAD could present a possible mechanism for the truncation of chemokines. Since many truncated chemokines have an increased biological activity, it would be interesting to examine if patients with CAD have more truncated chemokines compared to healthy subjects. In addition, it would be of interest to examine if truncated chemokines at local inflammatory sites interfere with the balance of extravasation and emigration and hence could contribute to leukocyte entrapment.

The skin blister fluid from patients with CAD induced the expression of CD36. Additional experiments are needed to explore if patients with CAD have an increased concentration of local factors with pro-atherosclerotic functions. This hypothesis needs to be further tested by incubating extravasated monocytes with their own chamber fluid *in vitro*. This enables a continued differentiation and discrimination of functional alterations in patients with CAD and healthy controls. Also, the potential factors in the skin chamber fluid needs to be identified. These include cytokines associated with differentiation such as IL-4 and GM-CSF.

Furthermore, the increased proportion of $CD14^{+}CD16^{+}$ monocytes at the local inflammatory site compared to in blood suggests a potential different role of this subtype compared to the $CD14^{+}CD16^{-}$ monocytes at an inflammatory location. It is not know today if the $CD14^{+}CD16^{+}$ monocytes have an increased migration to the skin chamber or if CD16 is induced following extravasation. The skin blister fluid did not influence the expression of CD16 but other mechanisms such as adhesion mediated activation could have potential effects [Li *et al* 2003]. The expression of CD16 could be examined following transwell mediated migration *in vitro*, following cross-linking of adhesion molecules and after incubation with activated endothelial cells.

Neutrophils from patients with CAD appear to have an altered activation profile. It would therefore be of interest to further evaluate activation of peripheral and extravasated neutrophils in patients with CAD and healthy subjects. Potential targets of

exploration include the composition of lipid rafts, PCK, PLC, Ca^{2+} mobilization and tyrosine phosphorylation.

Additional information is also needed on the expression of IL-1R1 following extravasation. The concentration of soluble IL-1R1 needs to be determined and the potential impact on phagocytosis has to be unraveled. The location of IL-1R1 in intracellular organelles and the increased surface expression following stimulation suggests IL-1R1 mobilization, however, this needs further studies.

Data from patients on medical interventions must always be thoroughly scrutinized. To include patients with limited interventions would of course be preferable but does not reflect the clinical reality. Many of the observed results could be influenced by statins which are endowed with several anti- inflammatory effects. *In vitro* experiments in the presence of statins are possible and could provide additional information of the potential impact of statins. However, a major obstacle that has to be solved is the hydroxylation and production of the active metabolite. Statins are processed by the liver into active metabolites and to incubate cells with intact statins would not truly reflect the biological activity.

In the best of world it would also be interesting to incubate the skin chamber over a longer period of time to allow potential modulation of the inflammatory milieu by extravasated neutrophils. In addition, if there were plenty of cells, co-cultures with extravasated monocytes and lymphocytes would be appealing to study.

7 SAMMANFATTNING PÅ SVENSKA

Immunförsvaret består av naiva och adaptiva försvarsmekanismer. Det naiva försvaret initieras direkt vid en infektion eller vävnadsskada och behöver ingen adaptiv fas. Två sorters celler är essentiella för denna försvarsmekanism, monocyter och neutrofiler. Dessa celler patrullerar blodbanan i jakt på mikroorganismer och eventuella skador. Monocyterna och neutrofilerna aktiveras vid en inflammation och transmigrerar från blodbanan ut i vävnaden. Transmigrationsprocessen regleras genom adhesionsmolekyler som CD11b och VLA-4 samt genom kemokiner från två huvudfamiljer: CXCL och CCL.

Arteriosklerotiska plack i de kärl som försörjer hjärtat med syre ger upphov till kranskärlssjukdom. Vid akuta tillstånd kan de arteriosklerotiska placken spricka vilket manifesteras som en hjärtinfarkt. Placken karakteriseras av en kronisk inflammation i kärlväggen som domineras av monocyter och mononukleära celler. Monocyter som transmigrerar till ett arteriosklerotiskt plack differentierar till makrofager. Makrofagerna kan fungera som antigenpresenterande celler och därigenom aktivera de adaptiva försvarscellerna som ytterligare bidrar till den inflammatoriska processen. De viktigaste komponenterna för antigenpresentation är HLA-komplexet som direkt binder till antigenet samt kostimulatoriska molekyler, som CD86, som förstärker cellsignaleringen. Makrofagerna tar även upp modifierat kolesterol, så kallat oxiderat LDL, och förvandlas därmed till fettinlagrade skumceller. De arteriosklerotiska placken ökar i storlek genom transmigration av monocyter och ackumulering av modifierat kolesterol. De receptorer som reglerar upptag av kolesterol kallas för scavenger-receptorer, CD36 är en av de viktigaste för utveckling av arterioskleros.

Neutrofiler är sällsynta i placken, dels för att en kronisk inflammation domineras av mononukleära celler och dels för att neutrofiler har ett kort livs-spann. Ett förhöjt antal neutrofiler i cirkulationen är dock starkt kopplat till en ökad risk för kranskärlssjukdom och neutrofiler kan ha en viktig immunomodulerande roll. Aktiverade neutrofiler producerar aktiva syreradikaler som bidrar till oxidering av kolesterol. Neutrofiler kan också producera kemokiner som bidrar till transmigration och enzymer som medverkar till att placken kan spricka.

I denna avhandling har vi använt en hudkammarmodell som gör det möjligt att studera celler som har transmigrerat från blodet till en lokal inflammation. Hudblåsor induceras på underarmen genom sugkoppar. Denna process lyfter det översta hudlagret så att det kan avlägsnas. Plastkoppar monteras sedan över såret och fylls med en buffertlösning eller serum. På detta sätt induceras en lokal inflammation i direkt anslutning till hudens kapillärbädd vilket möjliggör skördning av transmigrerade celler.

Syftet med avhandlingen var att studera transmigrerade monocyter och neutrofiler i patienter med kranskärlssjukdom och friska kontroller. Markörer kopplade till transmigrationsprocessen och till olika cellspecifika funktioner vid arterioskleros analyserades. Samtliga patienter hade haft en akut manifestation av sin sjukdom men var vid försökstillfället stabila och utan tecken på andra inflammatoriska tillstånd.

I **delarbete I och II** studerade vi transmigrerade monocyter. Patienter med kranskärlssjukdom hade en liknande inflammatorisk miljö och ett liknande antal celler i hudkammaren jämfört med friska kontroller. Efter transmigration uppvisade monocyter från patienter med kranskärlssjukdom ett lägre uttryck av adhesionsmolekylen CD11b. Övriga markörer associerade med transmigration av monocyter var inte förändrade. Ett förändrat uttryck av CD11b kan försvåra för monocyterna att transmigrera ut ur vävnaden och kan därmed bidra till ackumuleringen av monocyter. Markörer associerade med patologiska mekanismer vid kranskärlssjukdom var inducerade hos monocyter i hudkammaren i jämförelse med monocyter i blodet. Inga direkta skillnader påvisades mellan patienter och friska försökspersoner. Den inflammatoriska miljön från patienter med kranskärlssjukdom inducerade ett signifikant högre uttryck av CD36. Dessa fynd visar på att antigenpresentation och kolesterolupptag induceras vid eller strax efter transmigrationsprocessen och är lika för kranskärlspatienter och friska. Den lokala inflammatoriska miljön hos patienter med kranskärlssjukdom kan öka uttrycket av receptorer kopplade till kolesterolupptag.

I **delarbete III och IV** studerade vi transmigrerade neutrofiler. Transmigrerade neutrofiler från patienter med kranskärlssjukdom hade ett lägre uttryck av CD11b och en lägre produktion av reaktiva syremetaboliter efter stimulering. Data tyder på en förändrad reaktivitet och att neutrofiler hos patienter med kranskärlssjukdom kan vara refraktära mot starkare stimuleringar. Genanalys indikerade att transmigrerade neutrofiler har ett generellt högre yttryck av kemokingener och att IL-1 axeln kan vara viktig för cell aktivering. Transmigrerade neutrofiler från patienter med kranskärlssjukdom hade ett ökat uttryck av CCL20 och CXCL2 vilket kan bidra till ackumulering av mononukleära celler vid en lokal inflammation.

Vi har i denna avhandling påvisat en möjlig bidragande faktor till att monocyter ansamlas i arteriosklerotisk vävnad. Detta visades dels genom ett lägre uttryck av adhesionsmolekylen CD11b och dels genom att transmigrerade neutrofiler från patienter med kranskärlssjukdom hade en ökad produktion av vissa monocytrelaterade kemokiner. Vi har även påvisat att neutrofiler från patienter med kranskärlssjukdom har en förändrad aktivering och kan vara refraktära efter transmigration.

8 ACKNOWLEDGEMENTS

I would like to send my sincere gratitude to the following persons who have contributed to this thesis in so many different ways.

Joachim Lundahl, my main supervisor, thanks for believing in me and always pushing me one step forward. Your enthusiasm and endless ideas are the cornerstones of research, I appreciate your advises and new ways to scrutinize data. Finally, thanks for being such a friendly person.

Stefan Jacobson, my co-supervisor, thanks for your enthusiasm, good advices and your nice person. I have always felt comfort with your support.

Claes Held, my second co-supervisor, thanks for your expertise regarding cardiology.

Anette Bygden-Nylander, "Nessie", without you there had been no blisters, thanks for always being helpful and a genuine friend. Together we have solved the most strange problems, mysterious white powder, explosive glue and absence of cells, nothing could stop us!

My co-authors: *Ali Moshfegh*, you are such a good friend, thanks for your support when life as a PhD-student was tough. *Elham Dadfar*, my former room mate and student companion, thanks for good cooperation. I will remember our travels; go west is imprinted in my memory of a nice day along the Australian coast.

Former and current members of the inflammatory unit and the blister group:
Anna Nopp, it is good that you keep up the traditions with fika and Christmas lunches. Thanks for taking me "att luftas" and much laughter on the way. *Titti Nieminen*, who keeps the lab in order, I will remember our travels and lost sense of orientation. *Vladislav Muratov*, "Vladde", my room mate, you tell the most crazy and amusing stories, thanks for your uplifting serenades. *Elham Yektai*, I am glad to have shared life as a PhD-student with you; I wish you all the best. *Lena Wehlin*, you supported the new blue berry during the first year. *Britta Hylander*, thanks for sharing ideas at blister meetings and for being such a sense person. *Jenny Olsson* and *Carin Wahlqwist*, my PhD student companions, I wish you all the best, good luck with your own thesis. *Gerd Fransson Lundkvist*, you are always cheerful, thanks for the administrative support.

All friends and colleagues at L2:04, you are so many and I like you all!
I would specially like to thank *Helene*, for sharing the dissertation time with me, we manage to find the way trough all papers! Helene, *Erika* and *Christine*, I am happy that we encouraged each other to digest Janeway. *Tiiu*, *Patricia*, *Anna* and *Jeanette*, our trip out east was memorable; happy spirits in artic cold ☺. All the rest of L2:04, you mean a lot to me. It is comforting to know that whatever the time, there is always someone up for a fika! You provide the nice atmosphere and the cornerstones for a good research environment.

Min familj, ni betyder så mycket för mig:
Mamma och *Pappa*, tack för support och läxläsning med en envis liten flicka. *Viktor*, tänk att en lillebror kan vara så vuxen! Älskade *Mormor*, du tillför så mycket, tack vare dig kan jag både baka och virka. Mina *kusiner*, vad kul vi hade som barn, hänger ni med på burken i sommar? *Farbroder* och *morbroder* med familjer, vilken tur att ni finns.

Mina fantastiska vänner, jag är så tacksam för allt roligt vi hittar på:
Sevim och *Daniel* med familj, vi har många bra minnen tillsammans, tack för obegränsad vänskap och närvaro. Våra luncher är oersättliga, tänk vad mycket som kan avhandlas i stort och smått. *Johan*, det blir mycket jobbprat men vi har bra diskussioner och du förstår vikten av en fikastund. *Emma* med familj, vi har haft mycket kul med sångeriet. Kravallområdet i Paris var ju också något att minnas. Umgänget och månskärorna, tack för att jag kan lattja med er. *Matilda* och *Micke* med familj, ni är alldeles för trevliga för att bo så långt bort. Jag saknar spelkvällarna och småpratet över en kopp te. *Johanna*, på något sätt lyckas du vara lekfull och cool på samma gång. *Magnus*, du är ständigt full av upptåg och bus. *Sandra* och *Roger* med familj, jag förstår er härliga strävan efter en lantlig idyll. *Lars*, tänk att jag vågade dela tält med dig. *Ida* och *Thomas*, er brukar jag träffa på Gotland när det är dags för utflykter med BAD-bilen. Lagmedlemmar och vänner i *Ulvarna*, vilken tur att vi har en ”världsmästare som är bäst”. Jag har aldrig hållit på med något så gäckande men vänta bara, 2010 är mitt år!

9 REFERENCES

- Abram, C. L., and C. A. Lowell. 2009. The ins and outs of leukocyte integrin signaling. *Annu Rev Immunol* 27:339-362.
- Allavena, P., L. Piemonti, D. Longoni, S. Bernasconi, A. Stoppacciaro, L. Ruco, and A. Mantovani. 1998. IL-10 prevents the differentiation of monocytes to dendritic cells but promotes their maturation to macrophages. *Eur J Immunol* 28:359-369.
- Altznauer, F., S. Martinelli, S. Yousefi, C. Thurig, I. Schmid, E. M. Conway, M. H. Schoni, P. Vogt, C. Mueller, M. F. Fey, U. Zangemeister-Wittke, and H. U. Simon. 2004. Inflammation-associated cell cycle-independent block of apoptosis by survivin in terminally differentiated neutrophils. *J Exp Med* 199:1343-1354.
- Ancuta, P., K. Y. Liu, V. Misra, V. S. Wacleche, A. Gosselin, X. Zhou, and D. Gabuzda. 2009. Transcriptional profiling reveals developmental relationship and distinct biological functions of CD16+ and CD16- monocyte subsets. *BMC Genomics* 10:403.
- Ancuta, P., L. Weiss, and N. Haeffner-Cavaillon. 2000. CD14+CD16++ cells derived in vitro from peripheral blood monocytes exhibit phenotypic and functional dendritic cell-like characteristics. *Eur J Immunol* 30:1872-1883.
- Ancuta, P., R. Rao, A. Moses, A. Mehle, S. K. Shaw, F. W. Luscinskas, and D. Gabuzda. 2003. Fractalkine preferentially mediates arrest and migration of CD16+ monocytes. *J Exp Med* 197:1701-1707.
- Anderson, D. C., L. J. Miller, F. C. Schmalstieg, R. Rothlein, and T. A. Springer. 1986. Contributions of the Mac-1 glycoprotein family to adherence-dependent granulocyte functions: structure-function assessments employing subunit-specific monoclonal antibodies. *J Immunol* 137:15-27.
- Appelberg, R. 2007. Neutrophils and intracellular pathogens: beyond phagocytosis and killing. *Trends Microbiol* 15:87-92.
- Arend, W. P., M. Malyak, M. F. Smith, Jr., T. D. Whisenand, J. L. Slack, J. E. Sims, J. G. Giri, and S. K. Dower. 1994. Binding of IL-1 alpha, IL-1 beta, and IL-1 receptor antagonist by soluble IL-1 receptors and levels of soluble IL-1 receptors in synovial fluids. *J Immunol* 153:4766-4774.
- Baba, M., T. Imai, M. Nishimura, M. Kakizaki, S. Takagi, K. Hieshima, H. Nomiyama, and O. Yoshie. 1997. Identification of CCR6, the specific receptor for a novel lymphocyte-directed CC chemokine LARC. *J Biol Chem* 272:14893-14898.
- Baetta, R., and A. Corsini. 2009. Role of polymorphonuclear neutrophils in atherosclerosis: Current state and future perspectives. *Atherosclerosis*.
- Bainton, D. F. 1999. in Gallin, J. I. and Snyderman, *R. Inflammation : basic principles and clinical correlates.* Lippincott Williams & Wilkins, Philadelphia, PA.
- Bainton, D. F., J. L. Ullyot, and M. G. Farquhar. 1971. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *J Exp Med* 134:907-934.
- Barlic, J., Y. Zhang, and P. M. Murphy. 2007. Atherogenic lipids induce adhesion of human coronary artery smooth muscle cells to macrophages by up-regulating chemokine CX3CL1 on smooth muscle cells in a TNFalpha-NFkappaB-dependent manner. *J Biol Chem* 282:19167-19176.
- Bass, D. A., J. W. Parce, L. R. Dechatelet, P. Szejda, M. C. Seeds, and M. Thomas. 1983. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J Immunol* 130:1910-1917.

- Belge, K. U., F. Dayyani, A. Horelt, M. Siedlar, M. Frankenberger, B. Frankenberger, T. Espevik, and L. Ziegler-Heitbrock. 2002. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. *J Immunol* 168:3536-3542.
- Berahovich, R. D., Z. Miao, Y. Wang, B. Premack, M. C. Howard, and T. J. Schall. 2005. Proteolytic activation of alternative CCR1 ligands in inflammation. *J Immunol* 174:7341-7351.
- Berlin, C., R. F. Bargatze, J. J. Campbell, U. H. von Andrian, M. C. Szabo, S. R. Hasslen, R. D. Nelson, E. L. Berg, S. L. Erlandsen, and E. C. Butcher. 1995. alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell* 80:413-422.
- Berton, G., C. Laudanna, C. Sorio, and F. Rossi. 1992. Generation of signals activating neutrophil functions by leukocyte integrins: LFA-1 and gp150/95, but not CR3, are able to stimulate the respiratory burst of human neutrophils. *J Cell Biol* 116:1007-1017.
- Bevilacqua, M. P., J. S. Pober, D. L. Mendrick, R. S. Cotran, and M. A. Gimbrone, Jr. 1987. Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc Natl Acad Sci U S A* 84:9238-9242.
- Biasucci, L. M., G. D'Onofrio, G. Liuzzo, G. Zini, C. Monaco, G. Caligiuri, M. Tommasi, A. G. Rebuzzi, and A. Maseri. 1996. Intracellular neutrophil myeloperoxidase is reduced in unstable angina and acute myocardial infarction, but its reduction is not related to ischemia. *J Am Coll Cardiol* 27:611-616.
- Blankenberg, S., H. J. Rupprecht, O. Poirier, C. Bickel, M. Smieja, G. Hafner, J. Meyer, F. Cambien, and L. Tiret. 2003. Plasma concentrations and genetic variation of matrix metalloproteinase 9 and prognosis of patients with cardiovascular disease. *Circulation* 107:1579-1585.
- Boisvert, W. A., R. Santiago, L. K. Curtiss, and R. A. Terkeltaub. 1998. A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. *J Clin Invest* 101:353-363.
- Bonanno, E., A. Mauriello, A. Partenzi, L. Anemona, and L. G. Spagnoli. 2000. Flow cytometry analysis of atherosclerotic plaque cells from human carotids: a validation study. *Cytometry* 39:158-165.
- Borregaard, N., J. M. Heiple, E. R. Simons, and R. A. Clark. 1983. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. *J Cell Biol* 97:52-61.
- Borregaard, N., L. J. Miller, and T. A. Springer. 1987. Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils. *Science* 237:1204-1206.
- Borregaard, N., L. Kjeldsen, H. Sengelov, M. S. Diamond, T. A. Springer, H. C. Anderson, T. K. Kishimoto, and D. F. Bainton. 1994. Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and Mac-1 in human neutrophils during stimulation with inflammatory mediators. *J Leukoc Biol* 56:80-87.
- Brandolini, L., R. Bertini, C. Bizzarri, R. Sergi, G. Caselli, D. Zhou, M. Locati, and S. Sozzani. 1996. IL-1 beta primes IL-8-activated human neutrophils for elastase release, phospholipase D activity, and calcium flux. *J Leukoc Biol* 59:427-434.
- Browning, D. D., Z. K. Pan, E. R. Prossnitz, and R. D. Ye. 1997. Cell type- and developmental stage-specific activation of NF-kappaB by fMet-Leu-Phe in myeloid cells. *J Biol Chem* 272:7995-8001.
- Buono, C., H. Pang, Y. Uchida, P. Libby, A. H. Sharpe, and A. H. Lichtman. 2004. B7-1/B7-2 costimulation regulates plaque antigen-specific T-cell responses and atherogenesis in low-density lipoprotein receptor-deficient mice. *Circulation* 109:2009-2015.
- Campbell, J. J., E. F. Foxman, and E. C. Butcher. 1997. Chemoattractant receptor cross talk as a regulatory mechanism in leukocyte adhesion and migration. *Eur J Immunol* 27:2571-2578.

- Celie, J. W., R. H. Beelen, and J. van den Born. 2009. Heparan sulfate proteoglycans in extravasation: assisting leukocyte guidance. *Front Biosci* 14:4932-4949.
- Cheng, M., J. Wu, Y. Li, Y. Nie, and H. Chen. 2008. Activation of MAPK participates in low shear stress-induced IL-8 gene expression in endothelial cells. *Clin Biomech (Bristol, Avon)* 23 Suppl 1:S96-S103.
- Chomarat, P., J. Banchereau, J. Davoust, and A. K. Palucka. 2000. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol* 1:510-514.
- Chuluyan, H. E., and A. C. Issekutz. 1993. VLA-4 integrin can mediate CD11/CD18-independent transendothelial migration of human monocytes. *J Clin Invest* 92:2768-2777.
- Cloutier, A., T. Ear, E. Blais-Charron, C. M. Dubois, and P. P. McDonald. 2007. Differential involvement of NF-kappaB and MAP kinase pathways in the generation of inflammatory cytokines by human neutrophils. *J Leukoc Biol* 81:567-577.
- Coldren, C. D., J. A. Nick, K. R. Poch, M. D. Woolum, B. W. Fouty, J. M. O'Brien, M. P. Gruber, M. R. Zamora, D. Svetkauskaite, D. A. Richter, Q. He, J. S. Park, K. H. Overdier, E. Abraham, and M. W. Geraci. 2006. Functional and genomic changes induced by alveolar transmigration in human neutrophils. *Am J Physiol Lung Cell Mol Physiol* 291:L1267-1276.
- Condliffe, A. M., E. Kitchen, and E. R. Chilvers. 1998. Neutrophil priming: pathophysiological consequences and underlying mechanisms. *Clin Sci (Lond)* 94:461-471.
- Condliffe, A. M., E. R. Chilvers, C. Haslett, and I. Dransfield. 1996. Priming differentially regulates neutrophil adhesion molecule expression/function. *Immunology* 89:105-111.
- Conran, N., A. Gambero, H. H. Ferreira, E. Antunes, and G. de Nucci. 2003. Nitric oxide has a role in regulating VLA-4-integrin expression on the human neutrophil cell surface. *Biochem Pharmacol* 66:43-50.
- Couturier, C., N. Haeffner-Cavaillon, L. Weiss, E. Fischer, and M. D. Kazatchkine. 1990. Induction of cell-associated interleukin 1 through stimulation of the adhesion-promoting proteins LFA-1 (CD11a/CD18) and CR3 (CD11b/CD18) of human monocytes. *Eur J Immunol* 20:999-1005.
- Coxon, A., P. Rieu, F. J. Barkalow, S. Askari, A. H. Sharpe, U. H. von Andrian, M. A. Arnaout, and T. N. Mayadas. 1996. A novel role for the beta 2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. *Immunity* 5:653-666.
- Cushing, S. D., J. A. Berliner, A. J. Valente, M. C. Territo, M. Navab, F. Parhami, R. Gerrity, C. J. Schwartz, and A. M. Fogelman. 1990. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Natl Acad Sci U S A* 87:5134-5138.
- Dadfar, E., J. Lundahl, and S. H. Jacobson. 2004. Granulocyte extravasation and recruitment to sites of interstitial inflammation in patients with renal failure. *Am J Nephrol* 24:330-339.
- Dale, D. C., L. Boxer, and W. C. Liles. 2008. The phagocytes: neutrophils and monocytes. *Blood* 112:935-945.
- Damas, J. K., A. Boullier, T. Waehre, C. Smith, W. J. Sandberg, S. Green, P. Aukrust, and O. Quehenberger. 2005. Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, is elevated in coronary artery disease and is reduced during statin therapy. *Arterioscler Thromb Vasc Biol* 25:2567-2572.
- Davey, P. C., M. Zuzel, A. S. Kamiguti, J. A. Hunt, and K. A. Aziz. 2000. Activation-dependent proteolytic degradation of polymorphonuclear CD11b. *Br J Haematol* 111:934-942.
- de Boer, O. J., F. Hirsch, A. C. van der Wal, C. M. van der Loos, P. K. Das, and A. E. Becker. 1997. Costimulatory molecules in human atherosclerotic plaques: an indication of antigen specific T lymphocyte activation. *Atherosclerosis* 133:227-234.

- de Fougerolles, A. R., S. A. Stacker, R. Schwarting, and T. A. Springer. 1991. Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. *J Exp Med* 174:253-267.
- De, Y., Q. Chen, A. P. Schmidt, G. M. Anderson, J. M. Wang, J. Wooters, J. J. Oppenheim, and O. Chertov. 2000. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* 192:1069-1074.
- Diamond, M. S., D. E. Staunton, A. R. de Fougerolles, S. A. Stacker, J. Garcia-Aguilar, M. L. Hibbs, and T. A. Springer. 1990. ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18). *J Cell Biol* 111:3129-3139.
- Dopheide, J. F., U. Sester, A. Schlitt, G. Horstick, H. J. Rupprecht, T. Munzel, and S. Blankenberg. 2007. Monocyte-derived dendritic cells of patients with coronary artery disease show an increased expression of costimulatory molecules CD40, CD80 and CD86 in vitro. *Coron Artery Dis* 18:523-531.
- Draude, G., P. von Hundelshausen, M. Frankenberger, H. W. Ziegler-Heitbrock, and C. Weber. 1999. Distinct scavenger receptor expression and function in the human CD14(+)/CD16(+) monocyte subset. *Am J Physiol* 276:H1144-1149.
- Dunne, J. L., C. M. Ballantyne, A. L. Beaudet, and K. Ley. 2002. Control of leukocyte rolling velocity in TNF-alpha-induced inflammation by LFA-1 and Mac-1. *Blood* 99:336-341.
- Elbim, C., S. Bailly, S. Chollet-Martin, J. Hakim, and M. A. Gougerot-Pocidalo. 1994. Differential priming effects of proinflammatory cytokines on human neutrophil oxidative burst in response to bacterial N-formyl peptides. *Infect Immun* 62:2195-2201.
- Endemann, G., L. W. Stanton, K. S. Madden, C. M. Bryant, R. T. White, and A. A. Protter. 1993. CD36 is a receptor for oxidized low density lipoprotein. *J Biol Chem* 268:11811-11816.
- Eriksson, E. E., X. Xie, J. Werr, P. Thoren, and L. Lindbom. 2001. Direct viewing of atherosclerosis in vivo: plaque invasion by leukocytes is initiated by the endothelial selectins. *FASEB J* 15:1149-1157.
- Evans, B. J., A. McDowall, P. C. Taylor, N. Hogg, D. O. Haskard, and R. C. Landis. 2006. Shedding of lymphocyte function-associated antigen-1 (LFA-1) in a human inflammatory response. *Blood* 107:3593-3599.
- Fadok, V. A., M. L. Warner, D. L. Bratton, and P. M. Henson. 1998. CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor (alpha v beta 3). *J Immunol* 161:6250-6257.
- Faurschou, M., and N. Borregaard. 2003. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* 5:1317-1327.
- Fernandez, H. N., P. M. Henson, A. Otani, and T. E. Hugli. 1978. Chemotactic response to human C3a and C5a anaphylatoxins. I. Evaluation of C3a and C5a leukotaxis in vitro and under stimulated in vivo conditions. *J Immunol* 120:109-115.
- Follin, P., and C. Dahlgren. 2007. A skin chamber technique as a human model for studies of aseptic inflammatory reactions. *Methods Mol Biol* 412:333-346.
- Follin, P., M. P. Wymann, B. Dewald, M. Ceska, and C. Dahlgren. 1991. Human neutrophil migration into skin chambers is associated with production of NAP-1/IL8 and C5a. *Eur J Haematol* 47:71-76.
- Forsgren, A., and A. Scheja. 1985. Experience with a skin chamber technique for leucocyte migration studies. *Immunol Lett* 11:253-259.
- Fortun, A., A. Khalil, D. Gagne, N. Douziech, C. Kuntz, J. P. Jay-Gerin, G. Dupuis, and T. Fulop, Jr. 2001. Monocytes influence the fate of T cells challenged with oxidised low density lipoproteins towards apoptosis or MHC-restricted proliferation. *Atherosclerosis* 156:11-21.

- Franci, C., J. Gosling, C. L. Tsou, S. R. Coughlin, and I. F. Charo. 1996. Phosphorylation by a G protein-coupled kinase inhibits signaling and promotes internalization of the monocyte chemoattractant protein-1 receptor. Critical role of carboxyl-tail serines/threonines in receptor function. *J Immunol* 157:5606-5612.
- Fu, H., J. Bylund, A. Karlsson, S. Pellme, and C. Dahlgren. 2004. The mechanism for activation of the neutrophil NADPH-oxidase by the peptides formyl-Met-Leu-Phe and Trp-Lys-Tyr-Met-Val-Met differs from that for interleukin-8. *Immunology* 112:201-210.
- Fu, H., J. Karlsson, J. Bylund, C. Movitz, A. Karlsson, and C. Dahlgren. 2006. Ligand recognition and activation of formyl peptide receptors in neutrophils. *J Leukoc Biol* 79:247-256.
- Fuhler, G.M., N.R. Blom, P.J. Coffer, A.L. Drayer and E. Vellenga. 2007. The reduced GM-CSF priming of ROS production in granulocytes from patients with myelodysplasia is associated with an impaired lipid raft formation. *J Leukoc Biol.* 81:449-57.
- Fukuda, D., K. Shimada, A. Tanaka, T. Kusuyama, H. Yamashita, S. Ehara, Y. Nakamura, T. Kawarabayashi, H. Iida, M. Yoshiyama, and J. Yoshikawa. 2006. Comparison of levels of serum matrix metalloproteinase-9 in patients with acute myocardial infarction versus unstable angina pectoris versus stable angina pectoris. *Am J Cardiol* 97:175-180.
- Furie, M. B., and D. D. McHugh. 1989. Migration of neutrophils across endothelial monolayers is stimulated by treatment of the monolayers with interleukin-1 or tumor necrosis factor-alpha. *J Immunol* 143:3309-3317.
- Gaboury, J. P., and P. Kubes. 1994. Reductions in physiologic shear rates lead to CD11/CD18-dependent, selectin-independent leukocyte rolling in vivo. *Blood* 83:345-350.
- Gordon, S. 1999. in Gallin, J. I. and Snyderman, *R. Inflammation : basic principles and clinical correlates.* Lippincott Williams & Wilkins, Philadelphia, PA.
- Graham, I. L., H. D. Gresham, and E. J. Brown. 1989. An immobile subset of plasma membrane CD11b/CD18 (Mac-1) is involved in phagocytosis of targets recognized by multiple receptors. *J Immunol* 142:2352-2358.
- Greenberg, S. 1999. in Gallin, J. I. and Snyderman, *R. Inflammation : basic principles and clinical correlates.* Lippincott Williams & Wilkins, Philadelphia, PA.
- Gregory, A. D., L. A. Hogue, T. W. Ferkol, and D. C. Link. 2007. Regulation of systemic and local neutrophil responses by G-CSF during pulmonary Pseudomonas aeruginosa infection. *Blood* 109:3235-3243.
- Guichard, C., E. Pedruzzi, C. Dewas, M. Fay, C. Pouzet, M. Bens, A. Vandewalle, E. Ogier-Denis, M. A. Gougerot-Pocidalo, and C. Elbim. 2005. Interleukin-8-induced priming of neutrophil oxidative burst requires sequential recruitment of NADPH oxidase components into lipid rafts. *J Biol Chem* 280:37021-37032.
- Hachicha, M., P. Rathanaswami, P. H. Naccache, and S. R. McColl. 1998. Regulation of chemokine gene expression in human peripheral blood neutrophils phagocytosing microbial pathogens. *J Immunol* 160:449-454.
- Han, J., D. P. Hajjar, M. Febbraio, and A. C. Nicholson. 1997. Native and modified low density lipoproteins increase the functional expression of the macrophage class B scavenger receptor, CD36. *J Biol Chem* 272:21654-21659.
- Handberg, A., K. Levin, K. Hojlund, and H. Beck-Nielsen. 2006. Identification of the oxidized low-density lipoprotein scavenger receptor CD36 in plasma: a novel marker of insulin resistance. *Circulation* 114:1169-1176.
- Hansson, G. K., J. Holm, and L. Jonasson. 1989. Detection of activated T lymphocytes in the human atherosclerotic plaque. *Am J Pathol* 135:169-175.

- Hashmi, S., and Q. T. Zeng. 2006. Role of interleukin-17 and interleukin-17-induced cytokines interleukin-6 and interleukin-8 in unstable coronary artery disease. *Coron Artery Dis* 17:699-706.
- Haumer, M., J. Amighi, M. Exner, W. Mlekusch, S. Sabeti, O. Schlager, I. Schwarzinger, O. Wagner, E. Minar, and M. Schillinger. 2005. Association of neutrophils and future cardiovascular events in patients with peripheral artery disease. *J Vasc Surg* 41:610-617.
- Hechtman, D. H., M. I. Cybulsky, H. J. Fuchs, J. B. Baker, and M. A. Gimbrone, Jr. 1991. Intravascular IL-8. Inhibitor of polymorphonuclear leukocyte accumulation at sites of acute inflammation. *J Immunol* 147:883-892.
- Heine, G. H., C. Ulrich, E. Seibert, S. Seiler, J. Marell, B. Reichart, M. Krause, A. Schlitt, H. Kohler, and M. Girndt. 2008. CD14(++)CD16+ monocytes but not total monocyte numbers predict cardiovascular events in dialysis patients. *Kidney Int* 73:622-629.
- Heit, B., P. Colarusso, and P. Kubes. 2005. Fundamentally different roles for LFA-1, Mac-1 and alpha4-integrin in neutrophil chemotaxis. *J Cell Sci* 118:5205-5220.
- Heit, B., S. Tavener, E. Raharjo, and P. Kubes. 2002. An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. *J Cell Biol* 159:91-102.
- Hellum, K. B., and C. O. Solberg. 1977. Human leucocyte migration: studies with an improved skin chamber technique. *Acta Pathol Microbiol Scand C* 85C:413-423.
- Hemdahl, A. L., A. Gabrielsen, C. Zhu, P. Eriksson, U. Hedin, J. Kastrup, P. Thoren, and G. K. Hansson. 2006. Expression of neutrophil gelatinase-associated lipocalin in atherosclerosis and myocardial infarction. *Arterioscler Thromb Vasc Biol* 26:136-142.
- Hixenbaugh, E. A., Z. M. Goeckeler, N. N. Papaiya, R. B. Wysolmerski, S. C. Silverstein, and A. J. Huang. 1997. Stimulated neutrophils induce myosin light chain phosphorylation and isometric tension in endothelial cells. *Am J Physiol* 273:H981-988.
- Holmes, B., A. R. Page, and R. A. Good. 1967. Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocytic function. *J Clin Invest* 46:1422-1432.
- Horne, B. D., J. L. Anderson, J. M. John, A. Weaver, T. L. Bair, K. R. Jensen, D. G. Renlund, and J. B. Muhlestein. 2005. Which white blood cell subtypes predict increased cardiovascular risk? *J Am Coll Cardiol* 45:1638-1643.
- Huber-Lang, M., E. M. Younkin, J. V. Sarma, N. Riedemann, S. R. McGuire, K. T. Lu, R. Kunkel, J. G. Younger, F. S. Zetoune, and P. A. Ward. 2002. Generation of C5a by phagocytic cells. *Am J Pathol* 161:1849-1859.
- Huber-Lang, M., J. V. Sarma, F. S. Zetoune, D. Rittirsch, T. A. Neff, S. R. McGuire, J. D. Lambris, R. L. Warner, M. A. Flierl, L. M. Hoesel, F. Gebhard, J. G. Younger, S. M. Drouin, R. A. Wetsel, and P. A. Ward. 2006. Generation of C5a in the absence of C3: a new complement activation pathway. *Nat Med* 12:682-687.
- Huh, H. Y., S. F. Pearce, L. M. Yesner, J. L. Schindler, and R. L. Silverstein. 1996. Regulated expression of CD36 during monocyte-to-macrophage differentiation: potential role of CD36 in foam cell formation. *Blood* 87:2020-2028.
- Huh, H. Y., S. K. Lo, L. M. Yesner, and R. L. Silverstein. 1995. CD36 induction on human monocytes upon adhesion to tumor necrosis factor-activated endothelial cells. *J Biol Chem* 270:6267-6271.
- Imai, T., K. Hieshima, C. Haskell, M. Baba, M. Nagira, M. Nishimura, M. Kakizaki, S. Takagi, H. Nomiyama, T. J. Schall, and O. Yoshie. 1997. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell* 91:521-530.

- Inoue, H., M. Miyaji, A. Kosugi, M. Nagafuku, T. Okazaki, T. Mimori, R. Amakawa, S. Fukuhara, N. Domae, E. T. Bloom, and H. Umehara. 2002. Lipid rafts as the signaling scaffold for NK cell activation: tyrosine phosphorylation and association of LAT with phosphatidylinositol 3-kinase and phospholipase C-gamma following CD2 stimulation. *Eur J Immunol* 32:2188-2198.
- Inoue, T., H. Komoda, M. Nonaka, M. Kameda, T. Uchida, and K. Node. 2008. Interleukin-8 as an independent predictor of long-term clinical outcome in patients with coronary artery disease. *Int J Cardiol* 124:319-325.
- Jesaitis, A. J., E. S. Buescher, D. Harrison, M. T. Quinn, C. A. Parkos, S. Livesey, and J. Linner. 1990. Ultrastructural localization of cytochrome b in the membranes of resting and phagocytosing human granulocytes. *J Clin Invest* 85:821-835.
- Jiang, Y., J. F. Zhu, F. W. Luscinskas, and D. T. Graves. 1994. MCP-1-stimulated monocyte attachment to laminin is mediated by beta 2-integrins. *Am J Physiol* 267:C1112-1118.
- Johnson, Z., A. E. Proudfoot, and T. M. Handel. 2005. Interaction of chemokines and glycosaminoglycans: a new twist in the regulation of chemokine function with opportunities for therapeutic intervention. *Cytokine Growth Factor Rev* 16:625-636.
- Joiner, K. A., T. Ganz, J. Albert, and D. Rotrosen. 1989. The opsonizing ligand on Salmonella typhimurium influences incorporation of specific, but not azurophil, granule constituents into neutrophil phagosomes. *J Cell Biol* 109:2771-2782.
- Jonasson, L., J. Holm, O. Skalli, G. Bondjers, and G. K. Hansson. 1986. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis* 6:131-138.
- Karlsson, A., and C. Dahlgren. 2002. Assembly and activation of the neutrophil NADPH oxidase in granule membranes. *Antioxid Redox Signal* 4:49-60.
- Kiistala, U., and K. K. Mustakallio. 1964. In-Vivo Separation of Epidermis by Production of Suction Blisters. *Lancet* 1:1444-1445.
- Kiistala, U., and K. K. Mustakallio. 1967. Dermo-epidermal separation with suction. Electron microscopic and histochemical study of initial events of blistering on human skin. *J Invest Dermatol* 48:466-477.
- Kishimoto, T. K., K. O'Conner, and T. A. Springer. 1989. Leukocyte adhesion deficiency. Aberrant splicing of a conserved integrin sequence causes a moderate deficiency phenotype. *J Biol Chem* 264:3588-3595.
- Kitchen, E., A. G. Rossi, A. M. Condliffe, C. Haslett, and E. R. Chilvers. 1996. Demonstration of reversible priming of human neutrophils using platelet-activating factor. *Blood* 88:4330-4337.
- Kjeldsen, L., A. H. Johnsen, H. Sengelov, and N. Borregaard. 1993. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J Biol Chem* 268:10425-10432.
- Kjeldsen, L., D. F. Bainton, H. Sengelov, and N. Borregaard. 1994. Identification of neutrophil gelatinase-associated lipocalin as a novel matrix protein of specific granules in human neutrophils. *Blood* 83:799-807.
- Kjeldsen, L., H. Sengelov, K. Lollike, M. H. Nielsen, and N. Borregaard. 1994. Isolation and characterization of gelatinase granules from human neutrophils. *Blood* 83:1640-1649.
- Klebanoff, S. J. 1999. in Gallin, J. I. and Snyderman, *R. Inflammation : basic principles and clinical correlates.* Lippincott Williams & Wilkins, Philadelphia, PA.
- Klebanoff, S. J. 2005. Myeloperoxidase: friend and foe. *J Leukoc Biol* 77:598-625.
- Koivuranta-Vaara, P. 1985. Neutrophil migration in vivo: analysis of a skin window technique. *J Immunol Methods* 79:71-78.
- Kuhns, D. B., D. A. Long Priel, and J. I. Gallin. 1995. Loss of L-selectin (CD62L) on human neutrophils following exudation in vivo. *Cell Immunol* 164:306-310.

- Kuhns, D. B., E. DeCarlo, D. M. Hawk, and J. I. Gallin. 1992. Dynamics of the cellular and humoral components of the inflammatory response elicited in skin blisters in humans. *J Clin Invest* 89:1734-1740.
- Kunjathoor, V. V., M. Febbraio, E. A. Podrez, K. J. Moore, L. Andersson, S. Koehn, J. S. Rhee, R. Silverstein, H. F. Hoff, and M. W. Freeman. 2002. Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. *J Biol Chem* 277:49982-49988.
- Landsman, L., L. Bar-On, A. Zernecke, K. W. Kim, R. Krauthgamer, E. Shagdarsuren, S. A. Lira, I. L. Weissman, C. Weber, and S. Jung. 2009. CX3CR1 is required for monocyte homeostasis and atherogenesis by promoting cell survival. *Blood* 113:963-972.
- Lauber, K., E. Bohn, S. M. Krober, Y. J. Xiao, S. G. Blumenthal, R. K. Lindemann, P. Marini, C. Wiedig, A. Zobywalski, S. Baksh, Y. Xu, I. B. Autenrieth, K. Schulze-Osthoff, C. Belka, G. Stuhler, and S. Wesselborg. 2003. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell* 113:717-730.
- Laupeze, B., O. Fardel, M. Onno, N. Bertho, B. Drenou, R. Fauchet, and L. Amiot. 1999. Differential expression of major histocompatibility complex class Ia, Ib, and II molecules on monocytes-derived dendritic and macrophagic cells. *Hum Immunol* 60:591-597.
- Lei, Z. B., Z. Zhang, Q. Jing, Y. W. Qin, G. Pei, B. Z. Cao, and X. Y. Li. 2002. OxLDL upregulates CXCR2 expression in monocytes via scavenger receptors and activation of p38 mitogen-activated protein kinase. *Cardiovasc Res* 53:524-532.
- Lesnik, P., C. A. Haskell, and I. F. Charo. 2003. Decreased atherosclerosis in CX3CR1-/- mice reveals a role for fractalkine in atherogenesis. *J Clin Invest* 111:333-340.
- Lessner, S. M., H. L. Prado, E. K. Waller, and Z. S. Galis. 2002. Atherosclerotic lesions grow through recruitment and proliferation of circulating monocytes in a murine model. *Am J Pathol* 160:2145-2155.
- Ley, K., C. Laudanna, M. I. Cybulsky, and S. Nourshargh. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* 7:678-689.
- Li, G., G. Hangoc, and H. E. Broxmeyer. 2004. Interleukin-10 in combination with M-CSF and IL-4 contributes to development of the rare population of CD14+CD16++ cells derived from human monocytes. *Biochem Biophys Res Commun* 322:637-643.
- Li, G., Y. J. Kim, C. Mantel, and H. E. Broxmeyer. 2003. P-selectin enhances generation of CD14+CD16+ dendritic-like cells and inhibits macrophage maturation from human peripheral blood monocytes. *J Immunol* 171:669-677.
- Liles, W. C., J. A. Ledbetter, A. W. Waltersdorph, and S. J. Klebanoff. 1995. Cross-linking of CD18 primes human neutrophils for activation of the respiratory burst in response to specific stimuli: implications for adhesion-dependent physiological responses in neutrophils. *J Leukoc Biol* 58:690-697.
- Lindmark, E., L. Wallentin, and A. Siegbahn. 2001. Blood cell activation, coagulation, and inflammation in men and women with coronary artery disease. *Thromb Res* 103:249-259.
- Llodra, J., V. Angeli, J. Liu, E. Trogan, E. A. Fisher, and G. J. Randolph. 2004. Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. *Proc Natl Acad Sci U S A* 101:11779-11784.
- Loetscher, P., M. Seitz, I. Clark-Lewis, M. Baggiolini, and B. Moser. 1994. Both interleukin-8 receptors independently mediate chemotaxis. Jurkat cells transfected with IL-8R1 or IL-8R2 migrate in response to IL-8, GRO alpha and NAP-2. *FEBS Lett* 341:187-192.

- Lukacs, N. W., R. M. Strieter, V. Elner, H. L. Evanoff, M. D. Burdick, and S. L. Kunkel. 1995. Production of chemokines, interleukin-8 and monocyte chemoattractant protein-1, during monocyte: endothelial cell interactions. *Blood* 86:2767-2773.
- Lundqvist, H., P. Follin, L. Khalfan, and C. Dahlgren. 1996. Phorbol myristate acetate-induced NADPH oxidase activity in human neutrophils: only half the story has been told. *J Leukoc Biol* 59:270-279.
- Luo, Y., D. Jiang, D. Wen, J. Yang, and L. Li. 2004. Changes in serum interleukin-6 and high-sensitivity C-reactive protein levels in patients with acute coronary syndrome and their responses to simvastatin. *Heart Vessels* 19:257-262.
- Malcolm, K. C., P. G. Arndt, E. J. Manos, D. A. Jones, and G. S. Worthen. 2003. Microarray analysis of lipopolysaccharide-treated human neutrophils. *Am J Physiol Lung Cell Mol Physiol* 284:L663-670.
- Marshall, B. T., M. Long, J. W. Piper, T. Yago, R. P. McEver, and C. Zhu. 2003. Direct observation of catch bonds involving cell-adhesion molecules. *Nature* 423:190-193.
- Martinez, F. O., S. Gordon, M. Locati, and A. Mantovani. 2006. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* 177:7303-7311.
- McDonald, P. P., A. Bald, and M. A. Cassatella. 1997. Activation of the NF-kappaB pathway by inflammatory stimuli in human neutrophils. *Blood* 89:3421-3433.
- McDonald, P. P., and M. A. Cassatella. 1997. Activation of transcription factor NF-kappa B by phagocytic stimuli in human neutrophils. *FEBS Lett* 412:583-586.
- McEver, R. P., J. H. Beckstead, K. L. Moore, L. Marshall-Carlson, and D. F. Bainton. 1989. GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J Clin Invest* 84:92-99.
- Meisel, S. R., H. Shapiro, J. Radnay, Y. Neuman, A. R. Khaskia, N. Gruener, H. Pauzner, and D. David. 1998. Increased expression of neutrophil and monocyte adhesion molecules LFA-1 and Mac-1 and their ligand ICAM-1 and VLA-4 throughout the acute phase of myocardial infarction: possible implications for leukocyte aggregation and microvascular plugging. *J Am Coll Cardiol* 31:120-125.
- Melian, A., Y. J. Geng, G. K. Sukhova, P. Libby, and S. A. Porcelli. 1999. CD1 expression in human atherosclerosis. A potential mechanism for T cell activation by foam cells. *Am J Pathol* 155:775-786.
- Merched, A., K. Tollefson, and L. Chan. 2009. {beta}2 integrins modulate the initiation and progression of atherosclerosis in low-density lipoprotein receptor knockout mice. *Cardiovasc Res*.
- Millan, J., L. Hewlett, M. Glyn, D. Toomre, P. Clark, and A. J. Ridley. 2006. Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains. *Nat Cell Biol* 8:113-123.
- Miller, L. J., D. F. Bainton, N. Borregaard, and T. A. Springer. 1987. Stimulated mobilization of monocyte Mac-1 and p150,95 adhesion proteins from an intracellular vesicular compartment to the cell surface. *J Clin Invest* 80:535-544.
- Mizia-Stec, K., Z. Gasior, B. Zahorska-Markiewicz, J. Janowska, A. Szulc, E. Jastrzebska-Maj, and I. Kobielusz-Gembala. 2003. Serum tumour necrosis factor-alpha, interleukin-2 and interleukin-10 activation in stable angina and acute coronary syndromes. *Coron Artery Dis* 14:431-438.
- Mollinedo, F., M. Nakajima, A. Llorens, E. Barbosa, S. Callejo, C. Gajate, and A. Fabra. 1997. Major co-localization of the extracellular-matrix degradative enzymes heparanase and gelatinase in tertiary granules of human neutrophils. *Biochem J* 327 (Pt 3):917-923.

- Monk, P. N., A. M. Scola, P. Madala, and D. P. Fairlie. 2007. Function, structure and therapeutic potential of complement C5a receptors. *Br J Pharmacol* 152:429-448.
- Moser, B., M. Wolf, A. Walz, and P. Loetscher. 2004. Chemokines: multiple levels of leukocyte migration control. *Trends Immunol* 25:75-84.
- Mosig, S., K. Rennert, S. Krause, J. Kzhyshkowska, K. Neunubel, R. Heller, and H. Funke. 2009. Different functions of monocyte subsets in familial hypercholesterolemia: potential function of CD14+ CD16+ monocytes in detoxification of oxidized LDL. *FASEB J* 23:866-874.
- Mosser, D. M., and J. P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8:958-969.
- Muller, W. A., S. A. Weigl, X. Deng, and D. M. Phillips. 1993. PECAM-1 is required for transendothelial migration of leukocytes. *J Exp Med* 178:449-460.
- Nakata, A., Y. Nakagawa, M. Nishida, S. Nozaki, J. Miyagawa, T. Nakagawa, R. Tamura, K. Matsumoto, K. Kameda-Takemura, S. Yamashita, and Y. Matsuzawa. 1999. CD36, a novel receptor for oxidized low-density lipoproteins, is highly expressed on lipid-laden macrophages in human atherosclerotic aorta. *Arterioscler Thromb Vasc Biol* 19:1333-1339.
- Naruko, T., M. Ueda, K. Haze, A. C. van der Wal, C. M. van der Loos, A. Itoh, R. Komatsu, Y. Ikura, M. Ogami, Y. Shimada, S. Ehara, M. Yoshiyama, K. Takeuchi, J. Yoshikawa, and A. E. Becker. 2002. Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation* 106:2894-2900.
- Ndrepepa, G., S. Braun, J. Mehilli, N. von Beckerath, A. Schomig, and A. Kastrati. 2008. Myeloperoxidase level in patients with stable coronary artery disease and acute coronary syndromes. *Eur J Clin Invest* 38:90-96.
- Nelken, N. A., S. R. Coughlin, D. Gordon, and J. N. Wilcox. 1991. Monocyte chemoattractant protein-1 in human atheromatous plaques. *J Clin Invest* 88:1121-1127.
- Nijm, J., A. Wikby, A. Tompa, A. G. Olsson, and L. Jonasson. 2005. Circulating levels of proinflammatory cytokines and neutrophil-platelet aggregates in patients with coronary artery disease. *Am J Cardiol* 95:452-456.
- Nockher, W. A., and J. E. Scherberich. 1998. Expanded CD14+ CD16+ monocyte subpopulation in patients with acute and chronic infections undergoing hemodialysis. *Infect Immun* 66:2782-2790.
- Nopp, A., G. Hallden, J. Lundahl, E. Johansson, S. Vrtala, R. Valenta, R. Gronneberg, and M. Van Hage-Hamsten. 2000. Comparison of inflammatory responses to genetically engineered hypoallergenic derivatives of the major birch pollen allergen bet v 1 and to recombinant bet v 1 wild type in skin chamber fluids collected from birch pollen-allergic patients. *J Allergy Clin Immunol* 106:101-109.
- Ofek, I., and N. Sharon. 1988. Lectinophagocytosis: a molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. *Infect Immun* 56:539-547.
- Okamoto, Y., M. Tanaka, N. Miyahara, M. Tanabe, Y. Gotoh, T. Nagai, N. Fujita, T. Fukui, and T. Masuzawa. 2009. Age-dependent decreases in serum soluble interleukin-1 receptor type I (sIL-1RI) in healthy individuals: a population study of serum sIL-1RI levels in Japanese subjects. *J Clin Lab Anal* 23:175-178.
- Olofsson, P. S., Y. Sheikine, K. Jatta, M. Ghaderi, A. Samnegard, P. Eriksson, and A. Sirsjo. 2009. A functional interleukin-1 receptor antagonist polymorphism influences atherosclerosis development. The interleukin-1beta:interleukin-1 receptor antagonist balance in atherosclerosis. *Circ J* 73:1531-1536.
- Padrines, M., M. Wolf, A. Walz, and M. Baggiolini. 1994. Interleukin-8 processing by neutrophil elastase, cathepsin G and proteinase-3. *FEBS Lett* 352:231-235.

- Panaro, M. A., A. Acquafredda, M. Sisto, S. Lisi, A. B. Maffione, and V. Mitolo. 2006. Biological role of the N-formyl peptide receptors. *Immunopharmacol Immunotoxicol* 28:103-127.
- Park, Y. M., M. Febbraio, and R. L. Silverstein. 2009. CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima. *J Clin Invest* 119:136-145.
- Parker, K. P., W. R. Benjamin, K. L. Kaffka, and P. L. Kilian. 1989. Presence of IL-1 receptors on human and murine neutrophils. Relevance to IL-1-mediated effects in inflammation. *J Immunol* 142:537-542.
- Paschall, C. D., and M. B. Lawrence. 2008. L-selectin shear thresholding modulates leukocyte secondary capture. *Ann Biomed Eng* 36:622-631.
- Passlick, B., D. Flieger, and H. W. Ziegler-Heitbrock. 1989. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood* 74:2527-2534.
- Perillie, P. E., and S. C. Finch. 1964. Quantitative Studies of the Local Exudative Cellular Reaction in Acute Leukemia. *J Clin Invest* 43:425-430.
- Pober, J. S., and W. C. Sessa. 2007. Evolving functions of endothelial cells in inflammation. *Nat Rev Immunol* 7:803-815.
- Podrez, E. A., M. Febbraio, N. Sheibani, D. Schmitt, R. L. Silverstein, D. P. Hajjar, P. A. Cohen, W. A. Frazier, H. F. Hoff, and S. L. Hazen. 2000. Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species. *J Clin Invest* 105:1095-1108.
- Quie, P. G., J. G. White, B. Holmes, and R. A. Good. 1967. In vitro bactericidal capacity of human polymorphonuclear leukocytes: diminished activity in chronic granulomatous disease of childhood. *J Clin Invest* 46:668-679.
- Randolph, G. J. 2008. Emigration of monocyte-derived cells to lymph nodes during resolution of inflammation and its failure in atherosclerosis. *Curr Opin Lipidol* 19:462-468.
- Randolph, G. J., and M. B. Furie. 1996. Mononuclear phagocytes egress from an in vitro model of the vascular wall by migrating across endothelium in the basal to apical direction: role of intercellular adhesion molecule 1 and the CD11/CD18 integrins. *J Exp Med* 183:451-462.
- Ratnikov, B. I., A. W. Partridge, and M. H. Ginsberg. 2005. Integrin activation by talin. *J Thromb Haemost* 3:1783-1790.
- Richardson, R. M., H. Ali, E. D. Tomhave, B. Haribabu, and R. Snyderman. 1995. Cross-desensitization of chemoattractant receptors occurs at multiple levels. Evidence for a role for inhibition of phospholipase C activity. *J Biol Chem* 270:27829-27833.
- Robinson, J., F. Watson, R. C. Bucknall, and S. W. Edwards. 1992. Activation of neutrophil reactive-oxidant production by synovial fluid from patients with inflammatory joint disease. Soluble and insoluble immunoglobulin aggregates activate different pathways in primed and unprimed cells. *Biochem J* 286 (Pt 2):345-351.
- Rogacev, K. S., C. Ulrich, L. Blomer, F. Hornof, K. Oster, M. Ziegelin, B. Cremers, Y. Grenner, J. Geisel, A. Schlitt, H. Kohler, D. Fliser, M. Girndt, and G. H. Heine. 2009. Monocyte heterogeneity in obesity and subclinical atherosclerosis. *Eur Heart J*.
- Roos, D., R. S. Weening, A. A. Voetman, M. L. van Schaik, A. A. Bot, L. J. Meerhof, and J. A. Loos. 1979. Protection of phagocytic leukocytes by endogenous glutathione: studies in a family with glutathione reductase deficiency. *Blood* 53:851-866.
- Ruth, J. H., J. B. Rottman, K. J. Katschke, Jr., S. Qin, L. Wu, G. LaRosa, P. Ponath, R. M. Pope, and A. E. Koch. 2001. Selective lymphocyte chemokine receptor expression in the rheumatoid joint. *Arthritis Rheum* 44:2750-2760.
- Sainz, J., and M. Sata. 2008. Open sesame! CXCR4 blockade recruits neutrophils into the plaque. *Circ Res* 102:154-156.

- Salas, A., M. Shimaoka, A. N. Kogan, C. Harwood, U. H. von Andrian, and T. A. Springer. 2004. Rolling adhesion through an extended conformation of integrin alphaLbeta2 and relation to alpha I and beta I-like domain interaction. *Immunity* 20:393-406.
- Santin, A. D., P. L. Hermonat, A. Ravaggi, M. Chiriva-Internati, M. J. Cannon, J. C. Hiserodt, S. Pecorelli, and G. P. Parham. 1999. Expression of surface antigens during the differentiation of human dendritic cells vs macrophages from blood monocytes in vitro. *Immunobiology* 200:187-204.
- Sarndahl, E., I. Bergstrom, V. P. Brodin, J. Nijm, H. Lundqvist Setterud, and L. Jonasson. 2007. Neutrophil activation status in stable coronary artery disease. *PLoS One* 2:e1056.
- Scapini, P., J. A. Lapinet-Vera, S. Gasperini, F. Calzetti, F. Bazzoni, and M. A. Cassatella. 2000. The neutrophil as a cellular source of chemokines. *Immunol Rev* 177:195-203.
- Scheja, A., and A. Forsgren. 1985. A skin chamber technique for leukocyte migration studies; description and reproducibility. *Acta Pathol Microbiol Immunol Scand C* 93:25-30.
- Schiffmann, E., B. A. Corcoran, and S. M. Wahl. 1975. N-formylmethionyl peptides as chemoattractants for leucocytes. *Proc Natl Acad Sci U S A* 72:1059-1062.
- Schlenk, J., H. M. Lorenz, J. P. Haas, M. Herrmann, G. Hohenberger, J. R. Kalden, M. Rollinghoff, and H. U. Beuscher. 2005. Extravasation into synovial tissue induces CCL20 mRNA expression in polymorphonuclear neutrophils of patients with rheumatoid arthritis. *J Rheumatol* 32:2291-2298.
- Schlitt, A., G. H. Heine, S. Blankenberg, C. Espinola-Klein, J. F. Dopheide, C. Bickel, K. J. Lackner, M. Iz, J. Meyer, H. Darius, and H. J. Rupprecht. 2004. CD14+CD16+ monocytes in coronary artery disease and their relationship to serum TNF-alpha levels. *Thromb Haemost* 92:419-424.
- Selvatici, R., S. Falzarano, A. Mollica, and S. Spisani. 2006. Signal transduction pathways triggered by selective formylpeptide analogues in human neutrophils. *Eur J Pharmacol* 534:1-11.
- Semerad, C. L., F. Liu, A. D. Gregory, K. Stumpf, and D. C. Link. 2002. G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the blood. *Immunity* 17:413-423.
- Sengelov, H., F. Boulay, L. Kjeldsen, and N. Borregaard. 1994. Subcellular localization and translocation of the receptor for N-formylmethionyl-leucyl-phenylalanine in human neutrophils. *Biochem J* 299 (Pt 2):473-479.
- Sengelov, H., L. Kjeldsen, M. S. Diamond, T. A. Springer, and N. Borregaard. 1993. Subcellular localization and dynamics of Mac-1 (alpha m beta 2) in human neutrophils. *J Clin Invest* 92:1467-1476.
- Sengelov, H., P. Follin, L. Kjeldsen, K. Lollike, C. Dahlgren, and N. Borregaard. 1995. Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. *J Immunol* 154:4157-4165.
- Serrano, C. V., Jr., V. M. Yoshida, M. L. Venturinelli, E. D'Amico, H. P. Monteiro, J. A. Ramires, and P. L. da Luz. 2001. Effect of simvastatin on monocyte adhesion molecule expression in patients with hypercholesterolemia. *Atherosclerosis* 157:505-512.
- Shappell, S. B., C. Toman, D. C. Anderson, A. A. Taylor, M. L. Entman, and C. W. Smith. 1990. Mac-1 (CD11b/CD18) mediates adherence-dependent hydrogen peroxide production by human and canine neutrophils. *J Immunol* 144:2702-2711.
- Sheppard, F. R., M. R. Kelher, E. E. Moore, N. J. McLaughlin, A. Banerjee, and C. C. Silliman. 2005. Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. *J Leukoc Biol* 78:1025-1042.
- Skrzeczynska-Moncznik, J., M. Bzowska, S. Loseke, E. Grage-Griebenow, M. Zembala, and J. Pryjma. 2008. Peripheral blood CD14high CD16+ monocytes are main producers of IL-10. *Scand J Immunol* 67:152-159.

- Smith, C. W., S. D. Marlin, R. Rothlein, C. Toman, and D. C. Anderson. 1989. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. *J Clin Invest* 83:2008-2017.
- Smith, D. F., E. Galkina, K. Ley, and Y. Huo. 2005. GRO family chemokines are specialized for monocyte arrest from flow. *Am J Physiol Heart Circ Physiol* 289:H1976-1984.
- Soehnlein, O., A. Zernecke, E. E. Eriksson, A. G. Rothfuchs, C. T. Pham, H. Herwald, K. Bidzhekov, M. E. Rottenberg, C. Weber, and L. Lindbom. 2008. Neutrophil secretion products pave the way for inflammatory monocytes. *Blood* 112:1461-1471.
- Stanton, K. J., M. B. Frewin, and P. W. Gudewicz. 1999. Heterologous desensitization of IL-8-mediated chemotaxis in human neutrophils by a cell-binding fragment of fibronectin. *J Leukoc Biol* 65:515-522.
- Strzelecka, A., K. Kwiatkowska, and A. Sobota. 1997. Tyrosine phosphorylation and Fcgamma receptor-mediated phagocytosis. *FEBS Lett* 400:11-14.
- Suchard, S. J., M. J. Burton, and S. J. Stoehr. 1992. Thrombospondin receptor expression in human neutrophils coincides with the release of a subpopulation of specific granules. *Biochem J* 284 (Pt 2):513-520.
- Sudhakaran, P. R., A. Radhika, and S. S. Jacob. 2007. Monocyte macrophage differentiation in vitro: Fibronectin-dependent upregulation of certain macrophage-specific activities. *Glycoconj J* 24:49-55.
- Sun, H. W., C. J. Li, H. Q. Chen, H. L. Lin, H. X. Lv, Y. Zhang, and M. Zhang. 2007. Involvement of integrins, MAPK, and NF-kappaB in regulation of the shear stress-induced MMP-9 expression in endothelial cells. *Biochem Biophys Res Commun* 353:152-158.
- Takada, Y., X. Ye, and S. Simon. 2007. The integrins. *Genome Biol* 8:215.
- Takeshita, S., T. Isshiki, M. Ochiai, T. Ishikawa, Y. Nishiyama, T. Fusano, H. Toyoizumi, K. Kondo, Y. Ono, and T. Sato. 1997. Systemic inflammatory responses in acute coronary syndrome: increased activity observed in polymorphonuclear leukocytes but not T lymphocytes. *Atherosclerosis* 135:187-192.
- Tan, B. H., C. Meinken, M. Bastian, H. Bruns, A. Legaspi, M. T. Ochoa, S. R. Krutzik, B. R. Bloom, T. Ganz, R. L. Modlin, and S. Stenger. 2006. Macrophages acquire neutrophil granules for antimicrobial activity against intracellular pathogens. *J Immunol* 177:1864-1871.
- Tavora, F. R., M. Ripple, L. Li, and A. P. Burke. 2009. Monocytes and neutrophils expressing myeloperoxidase occur in fibrous caps and thrombi in unstable coronary plaques. *BMC Cardiovasc Disord* 9:27.
- Theilgaard-Monch, K., B. T. Porse, and N. Borregaard. 2006. Systems biology of neutrophil differentiation and immune response. *Curr Opin Immunol* 18:54-60.
- Theilgaard-Monch, K., S. Knudsen, P. Follin, and N. Borregaard. 2004. The transcriptional activation program of human neutrophils in skin lesions supports their important role in wound healing. *J Immunol* 172:7684-7693.
- Thelen, M. 2001. Dancing to the tune of chemokines. *Nat Immunol* 2:129-134.
- Thelen, M., and J. V. Stein. 2008. How chemokines invite leukocytes to dance. *Nat Immunol* 9:953-959.
- Thorburn, E., L. Kolesar, E. Brabcova, K. Petrickova, M. Petricek, M. Jaresova, A. Slavcev, and I. Striz. 2009. CXC and CC chemokines induced in human renal epithelial cells by inflammatory cytokines. *APMIS* 117:477-487.
- Tomhave, E. D., R. M. Richardson, J. R. Didsbury, L. Menard, R. Snyderman, and H. Ali. 1994. Cross-desensitization of receptors for peptide chemoattractants. Characterization of a new form of leukocyte regulation. *J Immunol* 153:3267-3275.
- Trillo, A. A. 1982. The cell population of aortic fatty streaks in African green monkeys with special reference to granulocytic cells. An ultrastructural study. *Atherosclerosis* 43:259-275.

- Uhing, R., and R. Snyderman. 1999. in Gallin, J. I. and Snyderman, *R. Inflammation : basic principles and clinical correlates.* Lippincott Williams & Wilkins, Philadelphia, PA.
- Umehara, H., E. T. Bloom, T. Okazaki, Y. Nagano, O. Yoshie, and T. Imai. 2004. Fractalkine in vascular biology: from basic research to clinical disease. *Arterioscler Thromb Vasc Biol* 24:34-40.
- van den Berg, J. M., F. P. Mul, E. Schippers, J. J. Weening, D. Roos, and T. W. Kuijpers. 2001. Beta1 integrin activation on human neutrophils promotes beta2 integrin-mediated adhesion to fibronectin. *Eur J Immunol* 31:276-284.
- Van den Steen, P. E., P. Proost, A. Wuyts, J. Van Damme, and G. Opdenakker. 2000. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood* 96:2673-2681.
- van Furth, R. 1985. Monocyte production during inflammation. *Comp Immunol Microbiol Infect Dis* 8:205-211.
- Veillard, N. R., V. Braunersreuther, C. Arnaud, F. Burger, G. Pelli, S. Steffens, and F. Mach. 2006. Simvastatin modulates chemokine and chemokine receptor expression by geranylgeranyl isoprenoid pathway in human endothelial cells and macrophages. *Atherosclerosis* 188:51-58.
- Voetman, A. A., and D. Roos. 1980. Endogenous catalase protects human blood phagocytes against oxidative damage by extracellularly generated hydrogen peroxide. *Blood* 56:846-852.
- Voisin, M. B., A. Woodfin, and S. Nourshargh. 2009. Monocytes and neutrophils exhibit both distinct and common mechanisms in penetrating the vascular basement membrane in vivo. *Arterioscler Thromb Vasc Biol* 29:1193-1199.
- Volger, O. L., J. O. Fledderus, N. Kisters, R. D. Fontijn, P. D. Moerland, J. Kuiper, T. J. van Berkel, A. P. Bijnens, M. J. Daemen, H. Pannekoek, and A. J. Horrevoets. 2007. Distinctive expression of chemokines and transforming growth factor-beta signaling in human arterial endothelium during atherosclerosis. *Am J Pathol* 171:326-337.
- Waehre, T., A. Yndestad, C. Smith, T. Haug, S. H. Tunheim, L. Gullestad, S. S. Froland, A. G. Semb, P. Aukrust, and J. K. Damas. 2004. Increased expression of interleukin-1 in coronary artery disease with downregulatory effects of HMG-CoA reductase inhibitors. *Circulation* 109:1966-1972.
- Waehre, T., J. K. Damas, L. Gullestad, A. M. Holm, T. R. Pedersen, K. E. Arnesen, H. Torsvik, S. S. Froland, A. G. Semb, and P. Aukrust. 2003. Hydroxymethylglutaryl coenzyme a reductase inhibitors down-regulate chemokines and chemokine receptors in patients with coronary artery disease. *J Am Coll Cardiol* 41:1460-1467.
- Wang, J. M., A. Sica, G. Peri, S. Walter, I. M. Padura, P. Libby, M. Ceska, I. Lindley, F. Colotta, and A. Mantovani. 1991. Expression of monocyte chemotactic protein and interleukin-8 by cytokine-activated human vascular smooth muscle cells. *Arterioscler Thromb* 11:1166-1174.
- Wang, Z. Q., A. S. Bapat, R. J. Rayanade, A. S. Dagtas, and M. K. Hoffmann. 2001. Interleukin-10 induces macrophage apoptosis and expression of CD16 (FcgammaRIII) whose engagement blocks the cell death programme and facilitates differentiation. *Immunology* 102:331-337.
- Werr, J., J. Johansson, E. E. Eriksson, P. Hedqvist, E. Ruoslahti, and L. Lindbom. 2000. Integrin alpha(2)beta(1) (VLA-2) is a principal receptor used by neutrophils for locomotion in extravascular tissue. *Blood* 95:1804-1809.
- Wesley, R. B., 2nd, X. Meng, D. Godin, and Z. S. Galis. 1998. Extracellular matrix modulates macrophage functions characteristic to atheroma: collagen type I enhances acquisition of resident macrophage traits by human peripheral blood monocytes in vitro. *Arterioscler Thromb Vasc Biol* 18:432-440.

- Williams, M. A., C. M. Cave, G. Quaid, C. Robinson, T. J. Daly, D. Witt, A. B. Lentsch, and J. S. Solomkin. 2005. Interleukin 8 dimerization as a mechanism for regulation of neutrophil adherence-dependent oxidant production. *Shock* 23:371-376.
- Wittmann, S., G. Rothe, G. Schmitz, and D. Frohlich. 2004. Cytokine upregulation of surface antigens correlates to the priming of the neutrophil oxidative burst response. *Cytometry A* 57:53-62.
- Wrann, C. D., S. W. Winter, T. Barkhausen, F. Hildebrand, C. Krettek, and N. C. Riedemann. 2007. Distinct involvement of p38-, ERK1/2 and PKC signaling pathways in C5a-mediated priming of oxidative burst in phagocytic cells. *Cell Immunol* 245:63-69.
- Wright, D. G., and J. I. Gallin. 1979. Secretory responses of human neutrophils: exocytosis of specific (secondary) granules by human neutrophils during adherence in vitro and during exudation in vivo. *J Immunol* 123:285-294.
- Yan, L., N. Borregaard, L. Kjeldsen, and M. A. Moses. 2001. The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL). Modulation of MMP-9 activity by NGAL. *J Biol Chem* 276:37258-37265.
- Yan, S. R., and M. J. Novak. 1999. Diverse effects of neutrophil integrin occupation on respiratory burst activation. *Cell Immunol* 195:119-126.
- Yano, R., M. Yamamura, K. Sunahori, K. Takasugi, J. Yamana, M. Kawashima, and H. Makino. 2007. Recruitment of CD16+ monocytes into synovial tissues is mediated by fractalkine and CX3CR1 in rheumatoid arthritis patients. *Acta Med Okayama* 61:89-98.
- Ye, R. D., S. L. Cavanagh, O. Quehenberger, E. R. Prossnitz, and C. G. Cochrane. 1992. Isolation of a cDNA that encodes a novel granulocyte N-formyl peptide receptor. *Biochem Biophys Res Commun* 184:582-589.
- Yesner, L. M., H. Y. Huh, S. F. Pearce and R. L. Silverstein. 1996. Regulation of monocyte CD36 and thrombospondin-1 expression by soluble mediators. *Arterioscler Thromb Vasc Biol* 16:1019-25.
- Zarbock, A., and K. Ley. 2008. Mechanisms and consequences of neutrophil interaction with the endothelium. *Am J Pathol* 172:1-7.
- Zeng, B., A. Prasan, K. C. Fung, V. Solanki, D. Bruce, S. B. Freedman, and D. Brieger. 2005. Elevated circulating levels of matrix metalloproteinase-9 and -2 in patients with symptomatic coronary artery disease. *Intern Med J* 35:331-335.
- Zernecke, A., I. Bot, Y. Djalali-Talab, E. Shagdarsuren, K. Bidzhekov, S. Meiler, R. Krohn, A. Schober, M. Sperandio, O. Soehnlein, J. Bornemann, F. Tacke, E. A. Biessen, and C. Weber. 2008. Protective role of CXC receptor 4/CXC ligand 12 unveils the importance of neutrophils in atherosclerosis. *Circ Res* 102:209-217.
- Ziegler-Heitbrock, H. W. 1996. Heterogeneity of human blood monocytes: the CD14+ CD16+ subpopulation. *Immunol Today* 17:424-428.
- Ziegler-Heitbrock, H. W., G. Fingerle, M. Strobel, W. Schraut, F. Stelter, C. Schutt, B. Passlick, and A. Pforte. 1993. The novel subset of CD14+/CD16+ blood monocytes exhibits features of tissue macrophages. *Eur J Immunol* 23:2053-2058.
- Ziegler-Heitbrock, L. 2007. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. *J Leukoc Biol* 81:584-592.
- Zimmerli, W., B. Seligmann, and J. I. Gallin. 1986. Exudation primes human and guinea pig neutrophils for subsequent responsiveness to the chemotactic peptide N-formylmethionylleucylphenylalanine and increases complement component C3bi receptor expression. *J Clin Invest* 77:925-933.